

UNIVERSIDADE FEDERAL DO PARANÁ

JESSICA ILKIW LOPES

LESÃO NA CAMADA GLOMERULAR DO BULBO OLFATÓRIO INTERFERE NA  
OLFAÇÃO E NOS COMPORTAMENTOS TIPO-DEPRESSIVOS EM MODELO  
ANIMAL DE PARKINSONISMO

CURITIBA

2017

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OLFAÇÃO E NOS COMPORTAMENTOS TIPO-DEPRESSIVOS EM MODELO  
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Dissertação apresentada como requisito parcial à obtenção do título de Mestre em Fisiologia, no Programa de Pós-Graduação em Fisiologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientador: Prof. Dr. Marcelo de Meira Santos Lima

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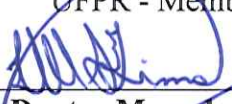
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## RESUMO

Os distúrbios olfatórios e a depressão são sintomas não-motores comumente relatados por indivíduos com a Doença de Parkinson (DP), sendo manifestados anos antes dos sintomas motores. Os mecanismos subjacentes a essas manifestações clínicas não estão completamente elucidados, no entanto, o desequilíbrio na neurotransmissão dopaminérgica parece ter um papel importante nesse contexto. Em indivíduos e em modelos animais da DP, é observado um aumento dos interneurônios dopaminérgicos da camada glomerular de bulbo olfatório (BO-gl), o que poderia contribuir para o prejuízo olfatório. Além disso, o desequilíbrio neuronal no BO está relacionado a sintomas depressivos, como demonstrado pela bulbectomia olfatória química. Dessa forma, nós hipotetizamos que a redução no número e/ou densidade dos neurônios dopaminérgicos presentes no BO poderia promover uma melhora olfatória e, em contrapartida, acentuaria o comportamento tipo-depressivo no modelo de Parkinsonismo induzido por 6-hidroxidopamina (6-OHDA). Assim, foi feita a administração intranigral de 6-OHDA e, posteriormente, a injeção da mesma droga na BO-gl em ratos, sendo esses avaliados em dois diferentes tempos (7 e 14 dias após a infusão intranigral de 6-OHDA). Observamos que, após 7 dias, o grupo lesionado apenas na SNpc apresentou comprometimento olfatório, assim como o grupo com a lesão apenas no BO. No entanto, a combinação das lesões na SNpc e BO foi capaz de reverter esse padrão, fazendo que os animais discriminassem ambos os compartimentos. Em relação ao comportamento tipo-depressivo, observamos que a lesão na SNpc promoveu comportamento tipo-depressivo, sendo esse acentuado após uma lesão dupla. A influência da SNpc e do BO no comportamento tipo-depressivo é corroborada por correlações entre parâmetros depressivos e neurônios TH-ir da SNpc em ambos tempos avaliados e com neurônios TH-ir do BO na avaliação de 14 dias. Esses resultados indicam um papel crucial do sistema dopaminérgico no BO e na SNpc tanto na modulação da olfação quanto no comportamento tipo-depressivo em um modelo de Parkinsonismo induzido por 6-OHDA.

**Palavras-chave:** Doença de Parkinson, depressão, distúrbios olfatórios, sistema dopaminérgico.

## ABSTRACT

Olfactory impairments and depressive behavior are commonly reported by individuals with Parkinson's disease (PD) being manifested before motor symptoms. The mechanisms underlying these clinical manifestations are not fully elucidated, however, the imbalance in dopaminergic neurotransmission seems to play an important role in the context. In individuals and animal models of PD, an increase in the dopaminergic interneurons of the glomerular layer in olfactory bulb (OB-gl) is observed, which could contribute to the olfactory impairment. In addition, neuronal imbalance in OB is related to depressive symptoms, as demonstrated by chemical olfactory bulbectomy. In view of that, we hypothesized that the reduction in the number and / or density of dopaminergic neurons present in OB could promote an olfactory improvement and, in contrast, would accentuate the depressive-like behavior in the 6-hydroxydopamine (6-OHDA) induced Parkinsonism model. Thereby, intranigral administration of 6-OHDA and subsequent injection of the same drug into OB-gl in rats were performed, being evaluated at two different time-points (7 and 14 days after intranigral infusion of 6-OHDA). We observed that, after 7 days, the group only injured in the SNpc presented olfactory impairment, as the group with the lesion only in the BO. However, the combination of the lesions in SNpc and BO was able to reverse this pattern, causing the animals to discriminate both compartments. In relation to depressive-like behaviors, we observed that the SNpc injury promoted depressive-like behavior, being accentuated after a double injury. The SNpc and OB influence on depressive-like behavior is corroborated by correlations between depressive parameters and SNpc TH-ir neurons at both evaluated times and with OB-gl TH-ir neurons in later time-point. These results indicate a crucial role of dopaminergic system in OB and SNpc in olfaction and humor modulation in a model of Parkinsonism induced by 6-OHDA.

**Keywords:** Parkinson's disease, depression, olfactory disorders, dopaminergic system.



## LISTA DE ABREVIATÖES

5-HT – 5-hidroxitriptamina – Serotonina  
6-OHDA – 6-hidroxidopamina  
AINE's – Anti-inflamatórios não esteroidais  
BO – Bulbo olfatório  
DA – Dopamina  
DAT – Transportador de dopamina  
DP – Doença de Parkinson  
EPI – Camada plexiforme externa  
GABA – Ácido gama-aminobutírico  
GI – Camada glomerular  
LC – *Locus coeruleus*  
MPP+ - 1-metil-4-phenylpyridinium  
MPTP - 1-metil-4-fenil-1,2,3,6-tetrahidropiridina  
NA - Noradrenalina  
OBX- Bulbectomia Olfatória Bilateral  
REM – Movimentos oculares rápidos  
ROS – Espécies reativas de oxigênio  
SN – Substância negra  
SNC – Sistema Nervoso Central  
SNpc – Substância negra pars compacta  
TDO – Tarefa de discriminação olfatória  
TH – Tirosina hidroxilase  
TH-ir – Neurônios imunorreativos à tirosina hidroxilase  
TNFM – Teste de natação forçada modificado  
TPS – Teste de preferência à sacarose  
VTA – Área tegmental ventral

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## 1. INTRODUÇÃO

### 1.1 DOENÇA DE PARKINSON

A doença de Parkinson (DP) é caracterizada por uma neurodegeneração progressiva e crônica, com uma taxa de incidência de 4,5-19 casos a cada 100.000 habitantes por ano (WHO, 2006) sendo a segunda doença neurodegenerativa progressiva mais prevalente (MHYRE *et al.*, 2012; DELAMARRE & MEISSNER, 2017). O aumento da idade é o maior fator de risco, afligindo cerca de 1% das pessoas com mais de 65 anos de idade e 4-5% das pessoas acima de 85 anos de idade (LANG & LOZANO, 1998; HIRSCH *et al.*, 2016). A incidência é 1,8 vezes maior em homens do que mulheres (WIRDEFELDT *et al.* 2011 *apud* MHYRE *et al.* 2012) e no Brasil – país que está em transição demográfica, apresentando diminuição da taxa de natalidade e aumento na longevidade (BOVOLENTA & FELICIO, 2017) – não há um número oficial sobre incidência da doença. Porém um estudo aponta a prevalência de 3,3% da população acima de 65 anos (BARBOSA *et al.*, 2006) – o que hoje representaria 630 mil pessoas (desconsiderando a população ≤64 anos) (BOVOLENTA & FELICIO, 2017)

A DP foi primeiramente descrita pelo médico britânico James Parkinson, em seu mais famoso artigo, “An essay on the shaking palsy”, de 1817, que a denominou “paralisia agitante”, já descrevendo os sinais motores clássicos como bradicinesia, rigidez, tremor de repouso e instabilidade postural (DAUER & PRZEDBORSKI, 2003). A mudança do nome para DP foi sugerida por Charcot, em homenagem a descrição clássica de Parkinson (TEIVE & MENEZES, 2003). Arvid Carlsson e colaboradores (1957) identificaram a dopamina como o neurotransmissor mais abundante nos gânglios basais e propuseram que a DP resultasse de depleção dopaminérgica, sendo essa hipótese demonstrada em estudos posteriores (CARLSSON *et al.* 1957; BERTLER & ROSENGREN, 1959). Além disso, foi descoberto que a reposição de dopamina por seu precursor levodopa (L-DOPA) ameniza os distúrbios motores (BIRKMAYER & HORNYKIEWICZ, 1961) – sendo utilizado até os dias de hoje (MHYRE *et al.* 2012). Entretanto, em função da cronicidade do tratamento com levodopa, observaram-se quadros de discinesia tardia manifestadas pelos pacientes, o que nos permite afirmar que este fármaco somente ameniza os sintomas, mas não impede a evolução da neurodegeneração –

além de não possuir efeito sobre outros sintomas relacionados à DP (DAUER & PRZEDBORSKI, 2003).

## 1.2 ETIOLOGIA DA DP

Menos de 10% dos casos da DP podem ser diretamente ligados às mutações genéticas (monogênicas e heterogênicas). Assim, fatores ambientais ou a combinação destes com possíveis susceptibilidades genéticas têm sido propostos como a possível causa da DP esporádica (TIEU, 2011). Desse modo, essa é vista como uma doença complexa, com múltiplos fatores genéticos e fatores de riscos ambientais associados (MHYRE *et al.* 2012). As causas específicas por trás da deflagração dos processos de degeneração que levam à doença são alvos de grande investigação na literatura (OSSOWSKA and LORENC-KOCI, 2013). Há evidências de que estresse oxidativo, disfunção mitocondrial, alterações conformacionais de proteínas, perturbações da homeostase intracelular de cálcio e ferro, além de polimorfismos em genes que regulam o metabolismo e o transporte de dopamina (DA), neuroinflamação e necrose/apoptose sejam possíveis fatores patológicos que desencadeiam a DP (LEWY *et al.*, 2009; LIMA *et al.*, 2012).

### 1.2.1 Estresse Oxidativo

O estresse oxidativo define um desequilíbrio entre os níveis de espécies reativas de oxigênio (ROS) produzidas e a capacidade de um sistema biológico para desintoxicar os intermediários reativos, criando um estado que contribui para danos celulares (DIAS *et al.*, 2013). Diversos estudos indicam que o dano oxidativo e a disfunção mitocondrial contribuem para a cascata de eventos que levam à degeneração dos neurônios dopaminérgicos da via nigroestriatal, responsável pelos sinais motores e não-motores da DP (SCHAPIRA and JENNER, 2011; ZHU and CHU, 2010; PARKER *et al.*, 2008; JENNER and OLANOW, 2006; BEAL, 2005).

São reconhecidas várias fontes e mecanismos para a geração de espécies reativas de oxigênio (ROS), incluindo o metabolismo da dopamina, disfunção mitocondrial, ferro, células neuroinflamatórias, cálcio e envelhecimento. Adicionalmente, os processos homeostáticos celulares incluindo o sistema de ubiquitina-proteassoma e a mitofagia são afetados pelo estresse oxidativo (DIAS *et al.*, 2013). A interação entre esses vários mecanismos contribui para a

neurodegeneração na DP como um cenário de retroalimentação positiva onde os danos primários levam ao estresse oxidativo, que danifica as principais proteínas patogênicas celulares que, por sua vez, causam mais produção de ROS (KIM *et al.*, 2015; DIAS *et al.*, 2013).

### 1.2.2 Neuroinflamação

Inflamação é um mecanismo de proteção no corpo que funciona para reparar, regenerar e remover os tecidos danificados / células ou agentes infecciosos, parasitas ou toxinas do corpo, onde respostas inflamatórias são realizadas por várias células imunológicas e inflamatórias, incluindo células T, neutrófilos, macrófagos, microglia e mastócitos (KULKARNI *et al.*, 2016). Do mesmo modo, a neuroinflamação é um mecanismo protetor para restaurar as células gliais danificadas e as células neuronais no sistema nervoso central. Ou seja, inicialmente a neuroinflamação é uma medida de proteção ao encéfalo, porém o excesso de respostas inflamatórias é prejudicial e de fato inibe a regeneração neuronal (KEMPURAJ *et al.*, 2016).

Fatores como o processo de envelhecimento normal, demência, trauma, acidente vascular cerebral, hipertensão, depressão, diabetes, tumores, infecções, toxinas e drogas podem iniciar a neuroinflamação no sistema nervoso central (SNC) (BARRIENTOS *et al.*, 2015). Além disso, o processo de envelhecimento normal causa diminuição da neurogênese, maior estresse metabólico, neuroinflamação aumentada, declínio cognitivo, déficits comportamentais e maior reatividade a qualquer desafio imunológico (DE VIRGILIO *et al.*, 2016; KEMPURAJ *et al.*, 2016). Ainda, estudos pré-clínicos demonstraram que o pré-tratamento com antiinflamatórios não-esteroidais (AINEs) protege os neurônios dopaminérgicos da degeneração induzida por neurotoxinas como 1-metil-4-fenil-1,2,5,6-tetraidropiridina (MPTP) e 6-hidroxidopamina (6-OHDA) (REKSIDLER *et al.*, 2007; BASSANI *et al.*, 2015) que induzem um importante aumento da expressão da enzima pró-inflamatória COX-2 (LIMA *et al.*, 2006).

### 1.2.3 Excitotoxicidade

A excitotoxicidade é definida como “(...) morte neuronal causada por altas concentrações de glutamato ou compostos com ação agonista sobre receptores glutamatérgicos” (OLNEY, 1969). O glutamato é o neurotransmissor excitatório mais abundante no encéfalo, sendo responsável por um terço de todas as sinapses do SNC (BARBOSA *et al.*, 2006 *apud* SANTIAGO *et al.*, 2014) e a excitotoxicidade ocorre devido a processos intracelulares pós hiperestimulação dos receptores glutamatérgicos ionotrópicos (AMPA e NMDA) responsáveis pelo influxo do íon  $\text{Ca}^{2+}$ . A maior entrada destes íons leva a ativação de fatores pró-apoptóticos e a produção de ROS, além de provocar dano mitocondrial (CAUDLE & ZHANG, 2009; AMBROSI *et al.*, 2014).

Um estudo mostrou a relação entre excitotoxicidade e DP ao estimularem um modelo de excitotoxicidade glutamatérgica em cultura celular e encontrarem a formação de aglomerados da proteína parkina (uma das proteínas ligada à formação de corpúsculos de Lewy) em mitocôndria e retículo endoplasmático (VAN LAAR *et al.*, 2015). Ainda, camundongos com parkinsonismo induzido por MPTP apresentaram aumento de glutamato extracelular quando comparado aos animais controles e animais MPTP tratados com L-DOPA apresentaram uma diminuição significativa de glutamato comparado aos animais MPTP não-tratados, o que pode indicar uma relação – possivelmente indireta – entre a neurodegeneração dopaminérgica e o aumento de glutamato extracelular, responsável pela excitotoxicidade (MEREDITH *et al.*, 2009).

### 1.2.4 Fatores Genéticos e Ambientais

Fatores genéticos têm sido identificados em formas Familiares de DP, o que compõe aproximadamente 10% dos casos. (LESAGE and BRICE, 2009; ROSNER and ORR-URTREGER, 2008). Os produtos de genes que causam a DP, incluindo DJ-1, PINK1, parkina, alfa-sinucleína e LRRK2, têm impacto em funções mitocondriais complexas, levando à exacerbação da geração de ROS e à susceptibilidade ao estresse oxidativo (DIAS *et al.*, 2013).

Enquanto os fatores genéticos podem explicar cerca de 60% dos casos de DP, sendo a maior parte esporádica, o que significa que há fatores ambientais e de

estilos de vida que influenciam o desenvolvimento da DP (MHYRE *et al.* 2012). O primeiro achado de fator ambiental foi reportado por Davis e colaboradores (1979), quando um paciente de 23 anos ao receber uma injeção intravenosa de análogos da Meperidina desenvolveu parkinsonismo. Pesquisas posteriores realizadas pelo grupo do Professor James Langston isolaram o composto e o identificaram como 1-metil-4-fenil-1,2,5,6-tetraidropiridina (MPTP), descobrindo-o como uma potente neurotoxina para os neurônios dopaminérgicos.

A partir da descoberta do MPTP, outra neurotoxina foi descoberta através de sua semelhança estrutural: o paraquat. Este é um herbicida muito utilizado na agricultura de todo mundo e é atualmente visto como um fator ambiental muito relevante (DAUER & PRZEDBORSKI, 2003). Além desse herbicida, há um pesticida utilizado em alguns países (como o Brasil) que também possui potencial neurotóxico: a rotenona (CICCHETTI *et al.*, 2009). Todos estes compostos atualmente são utilizados para mimetizar a DP em modelos animais (MHYRE *et al.* 2012).

### 1.3 MODELOS ANIMAIS NA PD

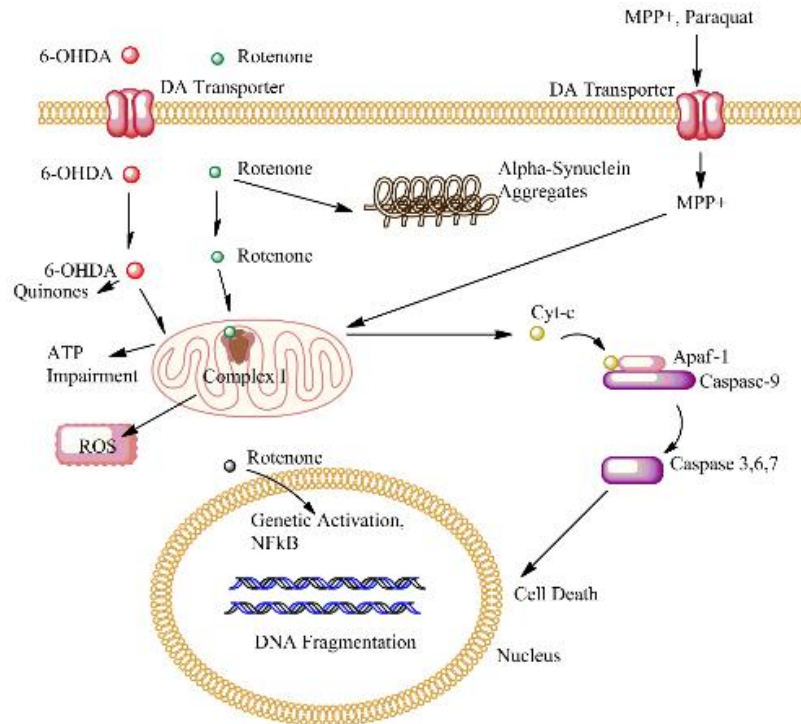
A partir dos diferentes fatores (ambientais e genéticos) que envolvem o surgimento da DP, modelos experimentais utilizando exposição a neurotoxinas exógenas, mutações nos genes ligados à DP ou uma combinação de ambos, são utilizados para estudar a DP e selecionar estratégias terapêuticas (TIEU, 2012). Os modelos animais atuais de DP podem ser divididos em duas categorias: modelos genéticos e neurotóxicos. Os modelos neurotóxicos envolvem a utilização de pesticidas, como paraquat e rotenona, e neurotoxinas, como MPTP e 6-OHDA, podendo ser utilizadas via intracerebral (CABEZAS *et al.*, 2013) e, no caso do MPTP, intranasal (PREDIGER *et al.*, 2006; PREDIGER *et al.*, 2009). Os mecanismos de neurotoxicidade destes agentes químicos estão representados abaixo (Fig.1).

Assim como MPTP, a 6-hidroxidopamina (6-OHDA) é uma neurotoxina catecolaminérgica seletiva utilizada em sua maioria para mimetizar a depleção dopaminérgica da via nigroestriatal em ratos (UNGERSTEDT, 1968). A lesão direta na SNpc para mimetizar a DP utilizando a 6-OHDA desencadeia seu efeito neurodegenerativo a partir de um mecanismo relacionado ao aumento do estresse oxidativo local (MEREDITH *et al.*, 2008; SANTIAGO *et al.*, 2014). Sendo

estruturalmente semelhante à DA, a 6-OHDA mostra uma elevada afinidade pelo transportador de DA (DAT), o que leva ao influxo da toxina nos neurônios dopaminérgicos, indicando haver grande seletividade de seus efeitos (LIMA *et al.*, 2009; SANTIAGO *et al.*, 2014). No neurônio, essa neurotoxina acumula-se no citosol e é submetida a uma rápida auto-oxidação, promovendo uma elevada taxa de formação de radicais livres, principalmente  $H_2O_2$ , e quinonas (MEREDITH *et al.*, 2008; LIMA *et al.*, 2009). Há evidências também de que a 6-OHDA age inibindo ativamente a cadeia respiratória mitocondrial à nível de complexo I (GLINKA and YODIM, 1995).

O modelo animal de 6-OHDA intranigral tem sido usado para testes pré-clínicos de novas terapias sintomáticas incluindo estratégias para prever alterações motoras e discinesias induzidas por levodopa (BOVÉ & PERIER, 2012). Os modelos animais não só replicam os sintomas motores da DP como também os sintomas não motores presentes em pacientes com a DP (ANSARI and JOHNSON, 1975; PREDIGER *et al.*, 2006). Em modelos animais, o comportamento tipo-depressivo é visto e comprovado através do aumento do tempo de imobilidade no teste de natação forçada e a diminuição do consumo de sacarose detectado no teste que quantifica a preferência pela solução açucarada, caracterizando o comportamento anedônico (SANTIAGO *et al.*, 2014). Já em relação aos distúrbios olfatórios na DP, sabe-se que os testes a serem realizados podem ter caráter sociais (odores que o animal convive/ poderá conviver) (MROCHEN *et al.*, 2016) e não sociais (odores que não são do convívio do animal), que são identificados e processados por diferentes vias olfatórias (FEINBERG *et al.*, 2012). Os odores usados em testes podem ser ainda prazerosos/ apetitosos – por exemplo, a apresentação de odor de urina de fêmea a machos (social) e odor de comida açucarada (não social) – ou aversivos, como odor de urina de predador (social) (LAZARINI *et al.*, 2014).

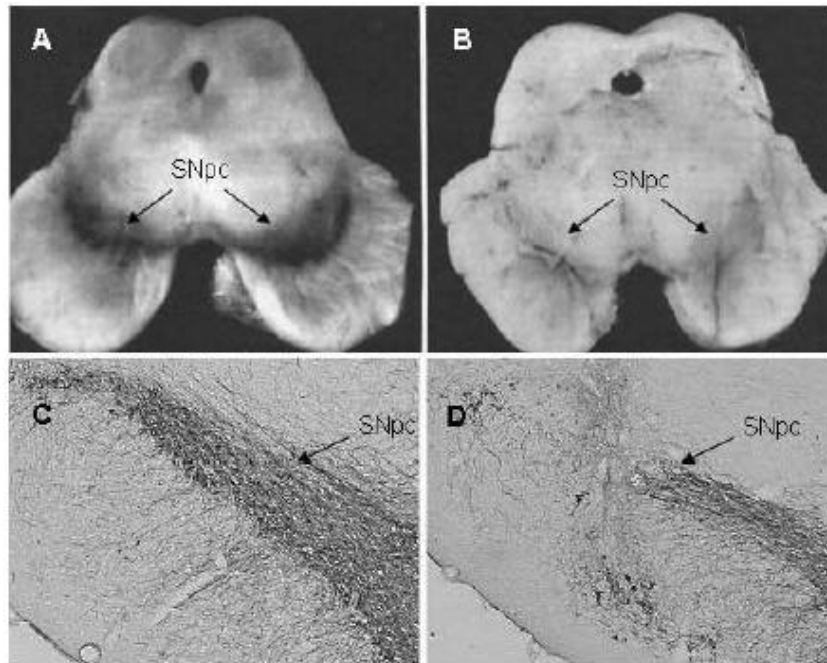




**Figura 1.** Modelos experimentais em PD. Paraquat, 6-hidroxidopamina (6-OHDA) e MPP+ (metabólito do MPTP) atravessam facilmente a membrana celular através do transportador de dopamina (DAT), induzindo assim a formação de agregados de  $\alpha$ -sinucleína e comprometimento mitocondrial com a subsequente produção de ROS e quinonas. Os compostos, como rotenona, são extremamente hidrofóbicos e penetram facilmente a membrana celular de neurônios e astrócitos. A rotenona pode promover processos tais como a formação de agregados de  $\alpha$ -sinucleína e a ativação genética através da translocação nuclear de NF- $\kappa$ B. Além disso, como inibidor do complexo mitocondrial I, a rotenona provoca o comprometimento do ATP, a geração de ROS e a liberação de moléculas pró-apoptóticas, como o citocromo c que ativam a caspase 9, que desencadeiam as caspases 3, 6 e 7 e induzem a apoptose. Retirado de Cabezas *et al.* 2013.

#### 1.4 FISIOPATOLOGIA DA DOENÇA DE PARKINSON

A fisiopatologia da DP envolve a degeneração progressiva de neurônios dopaminérgicos da substância negra pars compacta (SNpc) – evidenciada macroscopicamente pela despigmentação ventrolateral dessa estrutura (Fig. 2) – que desencadeia a denervação da via nigroestriatal e consequente redução significativa dos níveis de dopamina (DA) e seus metabólitos no estriado dorsal. Esse processo acarreta num desequilíbrio da projeção estriato-palidal e vias de saída do eixo palidal-talâmico, causando grande prejuízo motor (ALBIN, YOUNG & PENNEY, 1989; KANDEL, 2003).

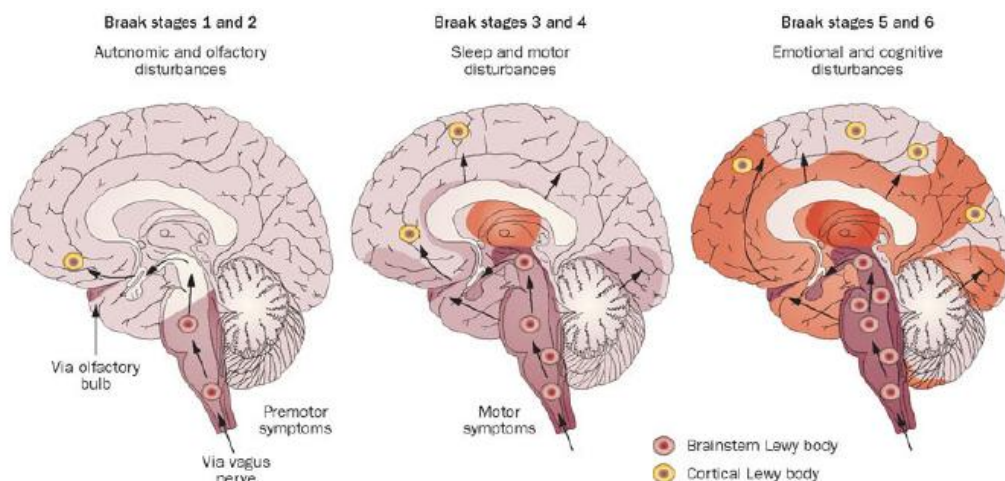


**Figura 2.** Perda maciça de neurônios dopaminérgicos do mesencéfalo ventral na substância negra pars compacta (SNpc). Os painéis A e B representam o mesencéfalo bilateral humano *postmortem* de indivíduos normais e parkinsonianos, respectivamente. C e D mostram o equivalente unilateral do mesencéfalo de rato depois de injeção intranigral do veículo (C) ou MPTP (D). As setas indicam a imunoreatividade da tirosina hidroxilase presente na SNpc. Retirado de Lima *et al.*, 2012.

A principal marca fisiopatológica da DP é a formação de inclusões intracitoplasmáticas protéicas nos neurônios dopaminérgicos, chamadas corpúsculos de Lewy (LBs) e neuritos de Lewy (LNs) que podem ser fusiformes ou em forma de fio. A  $\alpha$ -sinucleína é a principal proteína formadora desses corpúsculos, sendo estes também formados por parkina, ubiquitina, sinfilina-1, subunidades de proteossomas e várias outras proteínas (TRECIDI & BRAAK, 2012; OSSOWSKA & LORENC-KOCI, 2013).

O diagnóstico geralmente ocorre no início dos sintomas motores, porém, neste estágio, 40-60% dos neurônios dopaminérgicos da SNpc já foram acometidos (IRANZO, 2013; WALTER *et al.*, 2013). A fim de se compreender evolutivamente a progressão neuropatológica da DP, Braak e colaboradores (2004) estabeleceram seis estágios progressivos da doença, elaborando a hipótese de evolução ascendente da Doença de Parkinson (Fig. 3) (BRAAK *et al.*, 2004). No estágio 1 as primeiras lesões vistas no sistema nervoso central são no núcleo motor dorsal do nervo vago e no bulbo olfatório (BO). No estágio 2 os danos neuronais progridem ascendentemente, avançando ao longo do tegmento pedúnculo-pontino, afetando os

neurônios da porção mais baixa dos núcleos da rafe (serotoninérgico), porções magnocelulares da formação reticular e células noradrenérgicas do complexo *locus coeruleus* (LC) e *subcoeruleus* (este no caso de roedores). No estágio 3 ocorre as alterações na SNpc, células dopaminérgicas da amígdala central, neurônios colinérgicos do núcleo pedúnculo pontino, prosencéfalo basal e núcleos orais da rafe são também afetados. No estágio 4 ocorrem lesões nas áreas corticais do mesocórtex, este interligado com estruturas límbicas como: córtex entorrinal, amígdala e hipocampo. Nos estágios 5 e 6 ocorre a sobrecarga da SNpc devido à sua neurodegeneração e de todo o neocórtex, incluindo pré-frontal, associado à processos cognitivos (BRAAK *et al.*, 2004). Nos estágios 1 e 2 ocorre o aparecimento de prejuízos autonômicos e olfatórios; nos estágios 3 e 4 ocorrem distúrbios do sono e motores; e nos estágios 5 e 6 ocorrem os sintomas de distúrbios cognitivos e emocionais (DOTY, 2012).



**Figura 3.** O estágio esquemático da doença de Parkinson proposto por Braak, que mostra os locais de início no bulbo olfatório e medula oblonga, através do aparecimento de corpos de Lewy em regiões corticais. A patologia relacionada com  $\alpha$ -sinucleína é possivelmente iniciada na periferia através do epitélio olfatório ou do estômago, talvez envolvendo fatores de xenobióticos. O sombreamento vermelho representa o padrão topográfico da patologia. Retirado de Doty, 2012.

Desde sua descrição inicial em 1817 por James Parkinson, a DP é caracterizada por seus sinais motores como bradicinesia, rigidez, tremor de repouso, e instabilidade postural. Entretanto, os distúrbios não motores têm ganhado destaque na literatura como, por exemplo, as alterações olfatórias, de sono, no paladar, função cardiovascular, função gástrica e intestinal, salivação, atividade das glândulas sebáceas, humor e cognição (CHAUDHURI *et al.*, 2006; DOTY, 2013).

Alguns sintomas não motores como depressão, ansiedade, hiposmia, constipação e distúrbios de sono dos movimentos oculares rápidos (REM) podem aparecer antes dos distúrbios motores – chamada de fase prodrômica, onde o paciente apresenta os sintomas não motores, porém não pode ser diagnosticado já que os mesmos podem ser similares à outras enfermidades (LIU *et al.*, 2017). Outros sintomas como a demência, psicoses, sonolência e disfunções autonômicas, são muito comuns em estágios avançados (MODUGNO *et al.*, 2013).

Além da ligação de depressão e prejuízo olfatório à DP, diversos autores citam a relação entre depressão e prejuízo olfatório sem o contexto de Parkinsonismo (ATANASOVA *et al.*, 2008; NEGOLIAS *et al.*, 2010; ORAL *et al.*, 2013; CROY *et al.*, 2013; CROY *et al.*, 2014; YUAN & SLOTNICK, 2014; RAYNAUD *et al.*, 2015), pois é sabido que a depressão pode gerar prejuízo olfatório através da diminuição da neurogênese na zona subventricular – de onde neurônios migram para o BO – (CROY *et al.*, 2013; YUAN & SLOTNICK, 2014) o qual poderia ocorrer no BO, afetando os interneurônios granulares presentes na camada granular e os interneurônios periglomerulares presentes na camada glomerular (MARXREITER *et al.*, 2013), causando a redução direta da sensibilidade olfatória (NEGOLIAS *et al.*, 2010). Além disso, a bulbectomia olfatória bilateral (OBX) é considerada um modelo de depressão em animais, já que tanto a retirada cirúrgica quanto a lesão química bulbar acarretam alterações hipotalâmicas e límbicas (YUAN & SLOTNICK, 2014) similares às vistas em comportamentos tipo-depressivos e que podem ser responsáveis pelas alterações comportamentais, por exemplo, o aumento da hiperatividade em um ambiente novo, déficits de aprendizagem-evasão passiva, e anedonia (ORAL *et al.*, 2013; MATURANA *et al.*, 2014; RAYNAUD *et al.*, 2015).

#### 1.4.1 Depressão na DP

A depressão é uma comorbidade frequente na DP caracterizada principalmente por humor deprimido, perda de interesse e fadiga, sendo acompanhada por alterações encefálicas em sistema límbico e córtex pré-frontal (LIU *et al.*, 2012 *apud* CROY *et al.*, 2014), envolvendo o córtex orbito frontal, córtex cingulado anterior e posterior, ínsula, amígdala, hipocampo e tálamo (CROY *et al.*, 2014).

Considerada um sintoma não motor da DP, ocorre em 30-50% dos pacientes (DOBKIN *et al.*, 2011; AARSLAND *et al.*, 2012; ZAHODNE *et al.*, 2012; CHAGAS *et al.*, 2013; KETHARANATHAN *et al.*, 2014; FERNIE *et al.*, 2015). Os sintomas principais da depressão na DP são semelhantes aos observados no transtorno depressivo maior: humor deprimido e perda de interesse (VRIEND *et al.*, 2014). Estudos demonstraram que a depressão pode ser observada antes do aparecimento das alterações motoras (SHIBA *et al.*, 2000; MODUGNO *et al.*, 2013) e um diagnóstico prévio de transtorno depressivo maior está associado a um risco aumentado de desenvolvimento subsequente da DP (LEENTJENS *et al.*, 2003; SCHUURMAN *et al.*, 2002). Além disso, sabe-se que há maior prevalência de depressão relacionada à DP do que a outras doenças neurodegenerativas progressivas (AARSLAND *et al.*, 2012) e que a incidência de sintomas depressivos aumenta com a progressão da DP e a presumida disseminação da patologia (VRIEND *et al.*, 2014).

Desse modo, embora a depressão Parkinsoniana possa ter um componente reativo à descoberta do diagnóstico, evidências indicam que ela é causada pela depleção de DA, noradrenalina (NA) e serotonina (5-HT), ocorrendo desregulação de circuitarias fronto-subcorticais e límbicas que regulam o humor (MAYEUX, 1990; BLONDER *et al.*, 2013; CHAGAS *et al.*, 2013; OSSOWSKA e LORENC-KOCI, 2013; TUON *et al.*, 2014). Ou seja, a depressão, quando relacionada à DP, é uma consequência direta do processo neurodegenerativo, e não apenas uma reação emocional frente à doença crônica (ZAHODNE *et al.*, 2012; MARSH, 2013) já que ao longo da evolução da DP os neurônios noradrenérgicos do LC e serotoninérgicos dos núcleos da rafe são degenerados (ANTKIEWICZ-MICHALUK, 2002; BRAAK *et al.*, 2004), levando a diminuição dos níveis de 5-HT e NA nas regiões inervadas por estas estruturas. Ainda, não apenas os neurônios dopaminérgicos da SNpc são afetados, mas também – embora em menor grau – os da área tegmental ventral (ATV), que é a origem de projeções da via mesolímbica/mesocortical (OSSOWSKA & LORENC-KOCI, 2013). Além disso, foi encontrado que a neurodegeneração nigral é sete vezes maior em encéfalos post-mortem de pacientes com DP diagnosticados com depressão quando comparados aos encéfalos de pacientes DP sem esse transtorno de humor (FRISINA *et al.*, 2009).

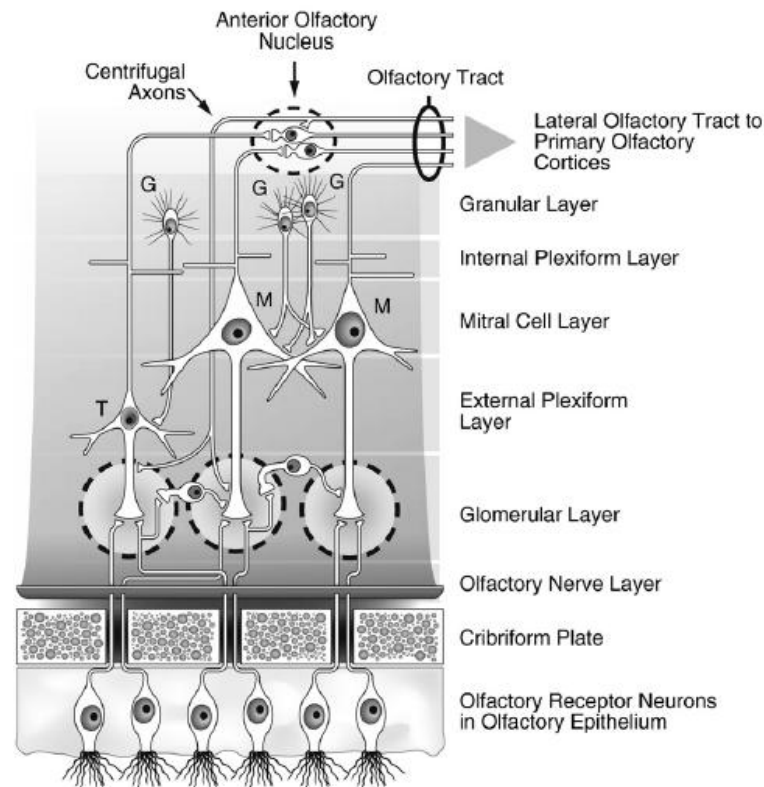
#### 1.4.2 Prejuízo Olfatório na DP

O prejuízo olfatório é talvez a primeira alteração pré-motora da DP (BRAAK *et al.*, 2003; DOTY, 2013), afetando mais de 90% dos pacientes (PREDIGER *et al.*, 2009; BERENDSE *et al.*, 2011; LAZARINI *et al.*, 2014; KNUDSEN *et al.*, 2015). Estudos realizados com parentes de pacientes têm sugerido a hiposmia como um sinal pré-motor que pode preceder o início dos sintomas motores em até cinco anos (BERENDSE *et al.*, 2011), porém outros estudos afirmam que o sintoma pode ser encontrado até décadas antes do primeiro sinal motor (DUDA, 2010). Em pacientes com DP foram encontradas evidências de corpos e neuritos de Lewy no bulbo olfatório, além de uma diminuição dos neurônios do núcleo olfatório anterior, bem como um aumento paradoxal de neurônios dopaminérgicos da camada glomerular do bulbo olfatório (interneurônios periglomerulares), mas a relação destes achados com o prejuízo olfatório ainda precisam ser elucidadas (LIU *et al.*, 2017).

O sistema olfatório de vertebrados é especializado em discriminar uma enorme variedade de moléculas e essa capacidade depende de uma série de etapas de processamento que ocorrem em diferentes estruturas ao longo do sistema olfatório: epitélio olfatório no nariz, bulbo olfatório e estruturas hierarquicamente superiores, tais como o córtex piriforme – que recebe informação proveniente do bulbo olfatório e a distribui para outras regiões do sistema nervoso (AIRES, 2012). Em humanos, essa via é única responsável por transmitir estímulos olfatórios (SALAZAR *et al.*, 2016). Já em roedores, o sistema olfatório é composto por duas vias distintas. A via previamente descrita é chamada de principal via olfatória enquanto a secundária é denominada via olfatória acessória (ou sistema vomeronasal) (SALAZAR *et al.*, 2016). As duas vias transmitem informações diferentes, a via principal transmitindo informações sobre estímulos olfatórios voláteis e a via acessória sobre não voláteis (MARTINEZ-MARCOS, 2009 *apud* FEINBERG *et al.*, 2012). Os odores sociais são compostos de uma variedade complexa de moléculas, além de componentes compartilhados entre os animais, transmitindo informações sobre a idade, sexo, estado de saúde e parentesco (BRENNAN & KENDRICK, 2006). Estes odores sociais são processados através de ambas as vias olfatórias, enquanto odores não sociais são processados unicamente através da via principal (FEINBERG *et al.*, 2012).

A transmissão do estímulo olfatório em humanos e pela via principal de roedores está representada na Figura 4. A molécula odorante é identificada pelos neurônios receptores olfatórios, presentes no epitélio olfatório do nariz. Esses neurônios projetam seus axônios para a camada mais externa do bulbo olfatório, (camada glomerular) onde o axônio dos neurônios receptores olfatórios encontram os dendritos dos neurônios de segunda ordem localizados no bulbo olfatório (células mitrales e tufochas m/t) em sinapses especializadas chamadas de glomérulos (KANDEL, 2003; AIRES, 2012).

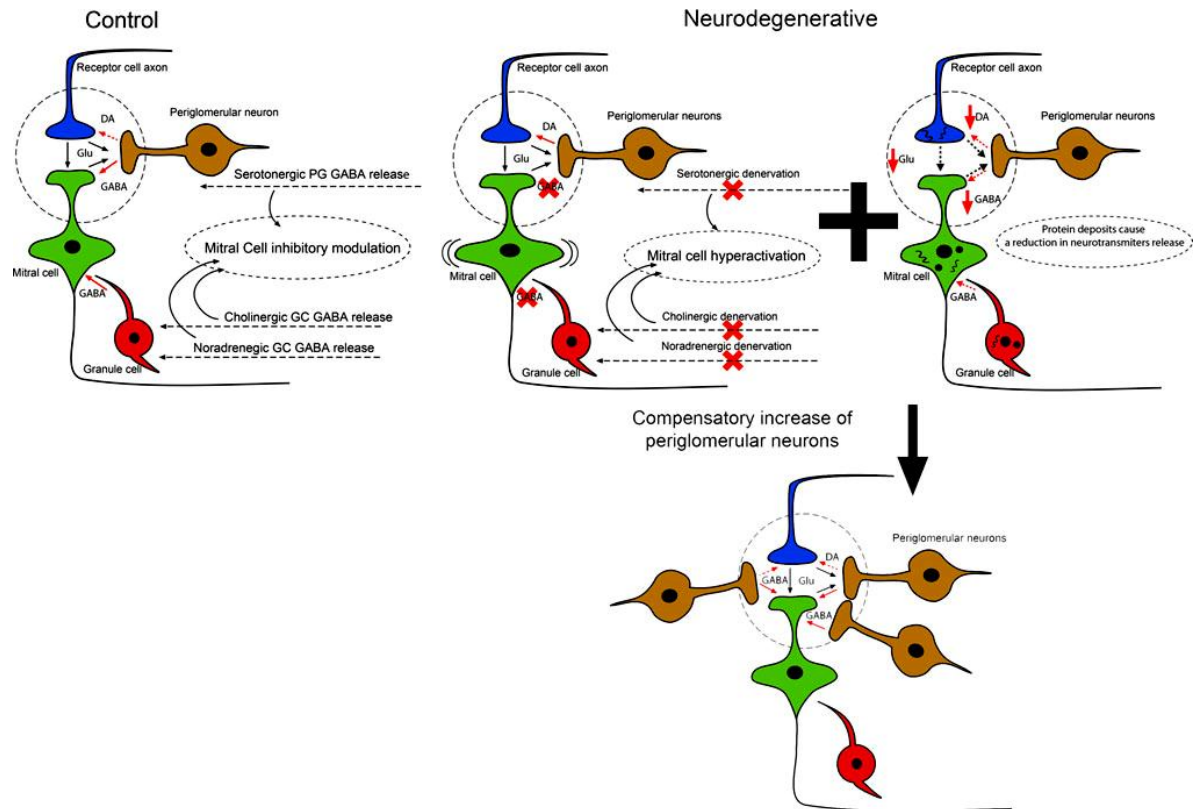
As células mitrales apresentam o corpo celular na camada mitral do bulbo olfatório, enquanto que as células tufochas apresentando seus corpos celulares na camada plexiforme externa (DUDA, 2010; KANDEL, 2003; DOTY, 2013). Há ainda a presença de interneurônios na camada glomerular (chamados de células periglomerulares) sendo estes em sua maioria neurônios dopaminérgicos que tem como função desempenhar uma ação inibitória sobre neurônios receptores olfatórios e células mitrales/tufochas (HUISMAN *et al.*, 2004; MUNDIÑANO *et al.*, 2011), modulando a transmissão do estímulo olfatório já que estas células possuem receptores dopaminérgicos da família D2 (inibitórios) (KANDEL, 2003; LAZARINI *et al.*, 2014).



**Figura 4.** Representação esquemática da circuitaria do bulbo olfatório. Neurônios receptores olfatórios projetam seus axônios para o epitélio através da placa crívica (cribriforme) que divergem ao entrar no bulbo olfatório para formar glomérulos. Nos glomérulos, os neurônios receptores olfatórios fazem sinapse com células mitrais (M) e células tufoosas (T) e são modulados pelos neurônios e axônios periglomerulares. Células mitrais e tufoosas são os principais neurônios do bulbo olfatório, projetando a informação olfatória para áreas corticais. Legenda: Olfactory receptor neurons (neurônios receptores olfatórios); cribriform plate (placa crivóide); olfactory nerve layer (camada de nervos olfatórios); glomerular layer (camada glomerular); external plexiform layer (camada plexiforme externa); mitral cell layer (camada de células mitrais); internal plexiform layer (camada plexiforme interna); granular layer (camada granular); lateral olfactory tract to primary olfactory cortices (trato olfatório lateral para o córtex olfatório primário); olfactory tract (trato olfatório); anterior olfactory nucleus (núcleo olfatório anterior); centrifugal axons (axônios centrifugos). Retirado de Duda, 2010.

Sabe-se que na DP há um aumento significativo do número destes interneurônios em humanos (HUISMAN *et al.*, 2004; MUNDIÑANO *et al.*, 2011) e também em modelo animal induzido pela rotenona (RODRIGUES *et al.*, 2014) – levantando a hipótese deste aumento ser uma resposta compensatória à perda de neurônios dopaminérgicos da SNpc – podendo ser relacionado ao prejuízo olfatório observado em tal condição (DOTY, 2012; DOTY, 2013). Mundiñano e colaboradores (2011), a partir dos resultados encontrados de aumento no número de neurônios dopaminérgicos na camada glomerular do BO, propuseram que tal ocorrência seja compensatória à diminuição das projeções serotoninérgicas e noradrenérgicas fruto da neurodegeneração de regiões como os núcleos da rafe e o LC durante as fases iniciais da DP (Fig. 5).





**Figura 5.** Representação esquemática da hipótese proposta por Mundiñano et al. (2011) para o aumento dos neurônios periglomerulares dopaminérgicos detectados na DP. Em situação normal (control), as aferências olfatórias colinérgicas, noradrenérgicas e serotoninérgicas exercem uma modulação inibitória das células mitrais. Nas doenças neurodegenerativas (neurodegenerative), as projeções colinérgicas, noradrenérgicas e serotoninérgicas estão reduzidas, o que pode diminuir ou mesmo extinguir estas projeções olfatórias centrífugas. O resultado desta diminuição seria a hiperatividade da célula mitral. Dado que os neurônios periglomerulares dopaminérgicos libertam neurotransmissores inibitórios tais como a dopamina (DA) e GABA para modular a sinalização olfatória no glomérulo, um aumento nos neurônios periglomerulares dopaminérgicos poderia ser uma resposta plástica para compensar a falta de inibição centrífuga. Por outro lado, os depósitos de Tau, amiloide e alfa-sinucleína nas células do bulbo olfatório podem afetar a liberação normal do neurotransmissor, reduzindo os níveis glomerulares de DA e GABA. O aumento observado de neurônios dopaminérgicos poderia ser uma resposta para compensar esse efeito. Retirado de Mundiñano et al., 2011.

A partir do achado de Huisman *et al.* (2004) e da proposição de Mundiñano *et al.* (2011), Rodrigues *et al.* (2014) identificaram um aumento de 50% dos interneurônios periglomerulares em ratos modelos de parkinsonismo induzido por rotenona intranigral quando comparado ao grupo controle. Desse modo, a hipótese do presente estudo é que a lesão com 6-OHDA infligida especificamente na camada glomerular do BO reduziria a quantidade de neurônios que estão aumentados devido à prévia lesão da via nigroestriatal – mimetizando a DP – que, hipoteticamente, seriam os responsáveis pela ocorrência dos distúrbios olfatórios. Portanto, a redução no número e/ou densidade destes neurônios dopaminérgicos presentes no BO

poderia promover uma melhora olfatória no contexto de Parkinsonismo induzido por 6-OHDA injetado na SNpc.

Há ainda uma hipótese alternativa que prevê que essa lesão do BO poderia gerar um prejuízo olfatório ainda maior (devido ao desequilíbrio neuronal causado pela lesão local), sendo averiguado através dos controles sham e do grupo que sofreu um bloqueio de detecção olfatória por meio da administração intranasal do fármaco Zicam<sup>®</sup>, previamente utilizado como controle positivo de anosmia, gerando o bloqueio da atividade dos neurônios receptores olfatórios presentes no epitélio olfatório, causando prejuízo olfatório clássico (LIM et al., 2009). Com relação às alterações tipo-depressivas, a hipótese levantada considera que os animais apresentem um comportamento tipo-depressivo – tanto comportamento de desamparo quanto anedonia – quando expostos à lesão dopaminérgica no BO frente exposição a 6-OHDA. Ou seja, a lesão intrabulbar poderia ter a ação de uma bulbectomia olfatória (OBX) e acentuar os sintomas depressivos já apresentados pelos animais lesionados na SNpc. Além disso, os animais controle positivo para anosmia (Zicam<sup>®</sup> intranasal) realizaram testes comportamentais para avaliação de parâmetros depressivos, com o intuito de identificar se uma lesão olfatória clássica seria capaz de induzir comportamento tipo-depressivo, e locomotores, para avaliação de possíveis danos motores.

## **2. JUSTIFICATIVA**

A depressão e os prejuízos olfatórios são importantes alterações não motoras encontradas na DP, levando os pacientes a uma piora significativa da qualidade de vida. Além disso, estas alterações são prodrômicas, o que as confere um alto valor preditivo para a DP. Deste modo, investigar os mecanismos fisiopatológicos e possíveis correlações entre essas disfunções, aparentemente distintas, torna-se essencial para que, através da identificação de biomarcadores moleculares e comportamentais, possa-se realizar um diagnóstico precoce e tratamento mais eficiente, além de expandir o entendimento funcional da circuitaria olfatória no contexto da regulação do humor.

### 3. OBJETIVOS

#### 3.1 OBJETIVO GERAL

Analisar os efeitos sobre a olfação, comportamentos tipo depressivos e motores causados pela lesão de neurônios dopaminérgicos da camada glomerular do bulbo olfatório mediante infusão local de 6-OHDA em ratos submetidos ao modelo de Parkinsonismo induzido por 6-OHDA na SNpc.

#### 3.2 OBJETIVOS ESPECÍFICOS

- Determinar a ocorrência de prejuízos motores, através do teste do campo aberto, nos animais submetidos ao paradigma de lesão dopaminérgica no BO e na via nigroestriatal.
- Determinar a ocorrência de prejuízos no desempenho olfatório, por meio do teste de discriminação olfatória, nos animais submetidos ao paradigma de lesão dopaminérgica no BO e na via nigroestriatal.
- Avaliar os comportamentos tipo-depressivos de desamparo, por meio do teste de natação forçada modificada, e de anedonia – teste de consumo de sacarose – nos animais submetidos ao paradigma de lesão dopaminérgica nigral e bulbar.
- Quantificação imuno-histoquímica da densidade e/ou número dos neurônios dopaminérgicos na SNpc e BO através da imuno-marcação para a enzima tirosina hidroxilase (TH).

#### 4. ARTIGO CIENTÍFICO

### **A selective lesion of periglomerular neurons in the olfactory bulb could restore olfaction and produce depressive-like behaviors in the 6-OHDA model of Parkinson's disease?**

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## Abstract

Olfactory impairments and depressive behavior are commonly reported by individuals with Parkinson's disease (PD) being manifested before motor symptoms. The mechanisms underlying these clinical manifestations are not fully elucidated, however, the imbalance in dopaminergic neurotransmission seems to play an important role in the context. In individuals and animal models of PD, an increase in the dopaminergic interneurons of the glomerular layer in olfactory bulb (OB-gl) is observed, which could contribute to the olfactory impairment. In addition, neuronal imbalance in OB is related to depressive symptoms, as demonstrated by chemical olfactory bulbectomy. In view of that, we hypothesized that the reduction in the number and / or density of dopaminergic neurons present in OB could promote an olfactory improvement and, in contrast, would accentuate the depressive-like behavior in the 6-hydroxydopamine (6-OHDA) induced Parkinsonism model. Thereby, intranigral administration of 6-OHDA and subsequent injection of the same drug into OB-gl in rats were performed, being evaluated at two different time-points (7 and 14 days after intranigral infusion of 6-OHDA). We observed that, after 7 days, the group only injured in the SNpc presented olfactory impairment, as the group with the lesion only in the BO. However, the combination of the lesions in SNpc and BO was able to reverse this pattern, causing the animals to discriminate both compartments. In relation to depressive-like behaviors, we observed that the SNpc injury promoted depressive-like behavior, being accentuated after a double injury. The SNpc and OB influence on depressive-like behavior is corroborated by correlations between depressive parameters and SNpc TH-ir neurons at both evaluated times and with OB-gl TH-ir neurons in later time-point. These results indicate a crucial role of dopaminergic system in OB and SNpc in olfaction and humor modulation in a model of Parkinsonism induced by 6-OHDA.

**Keywords:** Parkinson's disease, depression, olfactory disorders, dopaminergic system.

## Introduction

Parkinson's disease (PD) is the second most prevalent progressive neurodegenerative disease (Lang & Lozano, 1998; Pringsheim *et al.*, 2014), with an incidence rate of 4.5-19 cases per 100,000 inhabitants per year (WHO, 2006) and afflicting around 1% of people over 65 years old and 4-5% of those over 85 years old (Lang & Lozano, 1998). Since its discovery in 1817, PD is characterized by its motor signals such as bradykinesia, stiffness, resting tremor, and postural instability and its diagnosis usually occurs after the onset of motor symptoms, but at this stage, 40-60% of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) have already been degenerated (Iranzo, 2013; Walter *et al.*, 2013). Currently, non-motor disorders have been gaining increasing attention in the literature (Chaudhuri *et al.*, 2006; Doty, 2013). Since some non-motor disturbances such as depression, anxiety, hyposmia, constipation and rapid eyes movement (REM) sleep disorders appear before motor disorders (Modugno *et al.* 2013).

Olfactory impairment is the first pre-motor alteration of PD (Braak *et al.*, 2003; Doty, 2013), affecting more than 90% of patients (Berendse *et al.*, 2011, Lazarini *et al.*, 2014, Knudsen *et al.*, 2015). Studies with relatives of PD patients had established that hyposmia may precede motor symptoms in five years (Berendse *et al.*, 2011), but other studies affirm that this disturbance can be found decades before the motor onset (Duda, 2010). In fact, it has been reported a significant increase in the number of the dopaminergic periglomerular interneurons located within the glomerular layer of the olfactory bulb (OB), which are responsible for modulating olfactory transmission by inhibiting olfactory receptors cells and mitral/tufted neurons (Lazarini *et al.*, 2014) in both: humans (Huisman *et al.*, 2004; Mundiñano *et al.*, 2011) and rats (Rodrigues *et al.* 2014). Hence, this mechanism raises the hypothesis that this increased dopaminergic activity could be a compensatory response to the loss of dopaminergic neurons in the SNpc, possibly being related to olfactory impairment observed in such condition (Doty, 2012; Doty, 2013; Hölinger *et al.*, 2015).

Depression is a comorbidity that affects 30-50% of PD patients (Dobkin *et al.*, 2011, Aarsland *et al.*, 2012, Zahodne *et al.*, 2012, Chagas *et al.*, 2013; Ketharanathan *et al.*, 2014, Fernie *et al.*, 2015) and is characterized mainly by depressed mood, loss of interest and fatigue, being accompanied by encephalic alterations in the prefrontal limbic circuitry (Liu *et al.*, 2012; Croy *et al.*, 2014).

Depression symptoms are normally observed before motor alterations (Modugno et al., 2013) and it is known that there is a higher prevalence of PD-related depression than other progressive neurodegenerative diseases (Aarsland et al., 2012). Although, parkinsonian depression may have a reactive component, there are evidence that is caused by depletion of dopamine (DA), noradrenaline (NA) and serotonin (5-HT), with deregulation of frontal-subcortical and limbic circuitry (Blonder et al., 2013, Chagas et al., 2013, Ossowska and Lorenc-Koci, 2013, Tuon et al., 2014).

Interestingly, several reports have cited the relationship between depression and olfactory impairment without parkinsonism context (Atanasova et al., 2008, Negoias et al., 2010, Oral et al., 2013, Croy et al., 2014) Indeed, depression can cause olfactory impairment by decreasing neurogenesis in the subventricular zone consequently, preventing its migration to OB (Croy et al., 2013, Yuan and Slotnick, 2014) and also affecting granular and periglomerular interneurons activity (Marxreiter et al., 2013), causing direct reduction of olfactory sensitivity (Negoias et al., 2010). Accordingly, bilateral olfactory bulbectomy (OBX) is considered a model of depression in animals, since both surgical removal or chemical injury result in hypothalamic and limbic alterations (Yuan and Slotnick, 2014) such as depressive-like behaviors (Oral et al., 2013; Maturana et al., 2004; Raynaud et al., 2015). Furthermore, nigrostriatal lesions are strongly associated with remarkable increases of 50% and 100% of dopaminergic periglomerular neurons, in rats and PD patients, respectively, both negatively impacting olfaction (Rodrigues et al., 2014; Huisman et al., 2004; Huisman et al., 2011).

In view of that, the hypothesis of the present study is that a 6-hydroxydopamine (6-OHDA) lesion, inflicted specifically in the glomerular layer of OB, would prevent the compensatory increment of periglomerular neurons density generated by the 6-OHDA nigrostriatal lesion as an early phase model of PD. Therefore, we expect an olfactory improvement that could be related to the occurrence of depressive-like behaviors. Also, positive controls for anosmia (intranasal Zicam<sup>®</sup>) were tested for the parameters, in order to identify if a blockage of the olfactory epithelium (Lim et al., 2009; Chioca et al., 2011; Rodrigues et al., 2014) would be able to induce depressive-like and locomotor behaviors changes compatible with the hypothesis.



## Material and Methods

### *Ethics statement*

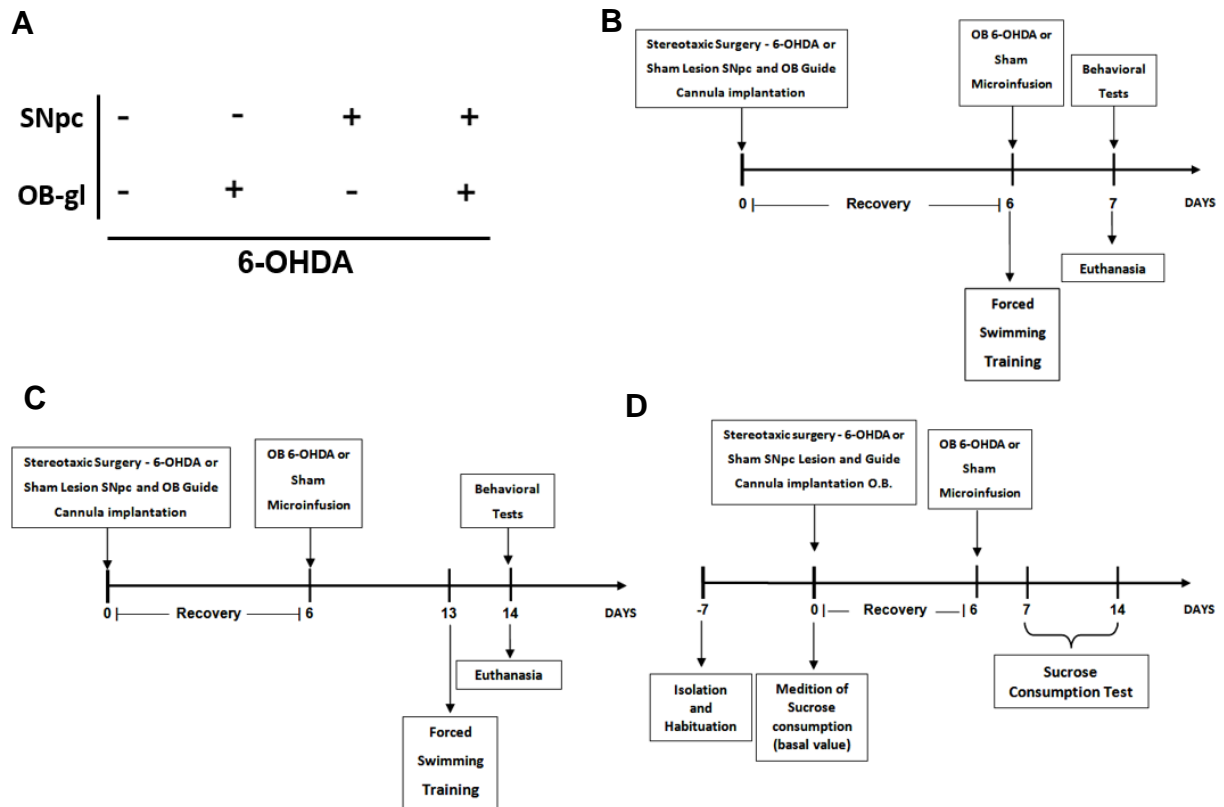
All the experiments were carried out in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals, United States National Institutes of Health. In addition, the protocol complies with the recommendations of Federal University of Paraná and was approved by the Institutional Ethics Committee (approval ID #910).

### *Animals*

Male Wistar rats from our breeding colony weighing 280–320 g at the beginning of the experiments were used. The animals were randomly housed in groups of five in polypropylene cages with wood shavings as bedding and maintained in a temperature-controlled room ( $22\pm 2^{\circ}\text{C}$ ) on a 12:12 h light-dark cycle (lights on at 7:00 AM). The animals had free access to water and food throughout the experiment.

### *Experimental design*

The animals were randomly distributed in four experimental groups (Fig. 1A): 6-OHDA (-)SNpc/(-)OB-gl; 6-OHDA (-)SNpc/(+)OB-gl; 6-OHDA (+)SNpc/(-)OB-gl; 6-OHDA (+)SNpc/(+)OB-gl. Signals (+) or (-) indicate infusion of 6-OHDA, or its vehicle, respectively. The experimental design shown in figure 2B indicates that on day zero the animals underwent stereotaxic infusion of bilateral 6-OHDA in the SNpc concomitantly to OB guide cannula implantation. Six days after, we performed microinfusions of 6-OHDA or vehicle within the OB. Afterwards, behavioral tests were performed according to the determined time-points: 7 and 14 days following SNpc lesions (Fig. 1B, 1C and 1D). In parallel, brain samples were collected 7 (Fig. 1B) and 14 days (Fig. 1C). Positive controls of anosmia were assigned as follows: 6-OHDA (+) SNpc / Zicam<sup>®</sup> (+) olfactory epithelium; 6-OHDA (-) SNpc / Zicam<sup>®</sup> (-) Olfactory epithelium. Zicam<sup>®</sup> damages the olfactory epithelium and causes olfactory impairment, according to previous data (Lim et al., 2009; Rodrigues *et al.*, 2014). The third experiment, represented in Figure 1D, shows the anedonic-like behaviors inflicted by the experimental conditions.



**Figure 1.** Experimental Design. **(A)** Representation of the four experimental groups (Total  $n=184$  animals, including positive controls groups),  $n=12$  per group for **B** and **C** experimental designs and  $n=8$  for **D** experimental design. Behavioral tests and brain samples collection were performed 7 **(B)** and 14 days **(C)** after intranigral 6-OHDA microinfusion. Both experiments also performed a second microinjection of 6-OHDA within the glomerular layer of the OB after the recovery period of 6 days. Also, at this time-point, a group of animals received intranasal infusion of Zicam, as a positive control of anosmia. **(D)** 14 days before surgery the animals were isolated for habituation. One week later one of the bottles had the water exchanged for 0.5% sucrose solution in order to measure the basal intake of sucrose before surgery. Surgery was performed on day 0 and the OB microinfusion on the sixth day after surgery, with the first measurement performed 7 days after intranigral 6-OHDA and the second 14 days after intranigral 6-OHDA. Legends: 6-OHDA, 6-hydroxydopamine; OB, olfactory bulb; SNpc, substantia nigra pars compacta.

### *Stereotaxic Surgery*

Rats were sedated with intraperitoneal xylazine (10 mg/kg; Syntec do Brasil Ltda, Brazil) and anaesthetized with intraperitoneal ketamine (90 mg/kg; Syntec do Brasil Ltda, Brazil). The following coordinates were used to the bilateral injury, bregma as a reference: substantia nigra pars compacta (SNpc) (AP) = - 5,0 mm, (ML) =  $\pm$  2,1 mm e (DV) = - 8,0 mm (Paxinos and Watson 2005). Needles were guided to the region of interest for a bilateral infusion of 2  $\mu$ L of 6-OHDA (3 $\mu$ g/ $\mu$ L) or of saline containing 0.2% ascorbic acid using an electronic infusion pump (Insight Instruments, Ribeirão Preto, Brazil) at a rate of 0,33  $\mu$ L/min for 6 minutes (modified from Lima, et al., 2006). Sham operations followed the same procedure, but 2  $\mu$ L of saline containing 0.02% ascorbic acid was injected instead. Complementarily, a

guide cannula was implanted in the olfactory bulb of each rat allowing a subsequent 1  $\mu\text{L}$  infusion of 6-OHDA (3  $\mu\text{g}/\mu\text{L}$ ) or vehicle (saline with 0,2% ascorbic acid) at a rate of 0,33  $\mu\text{L}/\text{min}$  for 3 minutes, in their respective groups. Coordinates with reference to bregma for implantation of guide cannula were: (AP) = +7.08 mm (ML) = 0.0 mm and (DV) = -3.6 mm (Paxinos and Watson 2005).

#### *Intranasal administration of Zicam (zinc gluconate + zinc acetate solution)*

The administration of Zicam<sup>®</sup> Oral Mist (Matrixx Initiatives, Scottsdale, AZ, USA) was performed as previously reported (Lim et al., 2009; Chioca *et al.*, 2013; Rodrigues et al., 2014): the animals were sedated with an intraperitoneal administration of 90 mg/kg ketamine and 3 mg/kg xylazine, and approximately 30  $\mu\text{L}$  of Zicam solution was slowly delivered into the nasal cavity using a Hamilton syringe connected to a blunted 30-gauge needle through a polyethylene tube. The polyethylene cannula was inserted 15 mm past the right external nostril to help irrigate the olfactory epithelium. The procedure was repeated in the left nostril. During respiration, part of the solution was expelled through the nostril and dried with absorbent paper to allow the animal to continue breathing.

#### *Olfactory discrimination task (ODT)*

This test was previously described by Soffié & Lamberty and subsequently modified by Prediger and colleagues (Soffié & Lamberty, 1988; Prediger et al., 2005a; Prediger et al., 2005b, Rodrigues *et al.*, 2014). The version used has been modified from Prediger et al., 2005a. The apparatus consisted of a box (60 x 40 x 50cm) equally divided into two compartments, connected by a door that gives free access to the animal. Before the test, it was performed an adaptation period to the apparatus of 2 minutes, in both compartments with fresh sawdust. After that, clean sawdust is added on one side of the box (non-familiar odor). On the other side of the box, is added sawdust which animals remained isolated for 48 hours before testing (familiar odor). The ODT consists of placing the rat in the middle of olfactory discrimination box and record, up to 3 min, the time of investigation of each compartment. The animal that shows olfactory impairment tends to explore both compartments equally, indicating absence of discrimination. As a measure of discrimination, a “discrimination index (DI)” was calculated by dividing the difference

in exploration time between the two compartments (compartment non-familiar - compartment familiar) by the total amount of exploration for both compartments (compartment non-familiar + compartment familiar). DI was then multiplied by 100 to express it as a percentage.

#### *Modified forced swimming test (MFST)*

This test is a modified version of Detke *et al.* (1995) e Cryan *et al.* (2002). Rats were placed in an opaque plastic cylinder (diameter 20 cm; height 50 cm) containing water up to 30 cm ( $24 \pm 1^\circ\text{C}$ ); on day 1 the rats remained in the cylinder for 15 min (training session) and 24h later they were placed back and tested for 5-min (test session). The test session was video recorded via a camera positioned above the cylinder for subsequent analysis. The behaviors registered during the test session were: immobility (when the rat stopped all active behaviors and remained floating in the water with minimal movements, with its head just above the water), swimming (movements through out the swim cylinder, including crossing into another quadrant) and climbing (upward movements of the forepaws along the cylinder walls). The time spend in each one of the behavior was analyzed. The water was changed and the cylinder rinsed with clean water after each rat. Following the training and the test sessions, the animals were dried and placed in their home cages.

#### *Open-Field Test (OF)*

The apparatus consists of a circular arena (1 m of diameter) limited by a 40-cm-high wall and illuminated by four 60-W lamps situated 100 cm above the arena floor, providing illumination around 300lx (Broadhurst, 1960). The animals were gently placed in the center of the arena and were allowed to freely explore the area for 5 min. During the experiments, the open-field was video recorded and the measures for travelled distance and computed online by an image analyzer (Smart Junior, PanLab, Harvard Apparatus, Spain).

#### *Sucrose Preference Test (SPT)*

Sucrose preference is frequently used as measure of anhedonia (Papp *et al.*, 1991; Wang *et al.*, 2009; Santiago *et al.*, 2014). Eight days before intranigral 6-OHDA the animals were transferred to single-housing cages with free access to food. Each rat was provided with two preweighed bottles of water on opposite sides of the cage during 24h for the training phase to adapt the rats to drinking from two bottles. After training, one bottle was randomly switched to contain a 0.5% sucrose solution as described previously (Slattery *et al.*, 2007; Martynhak *et al.*, 2015). Two days later, the bottles were reversed to avoid perseveration effects. The bottles were weighed before being offered to the animal and at the end of the experiment (one week later). The sum of water consumption and sucrose consumption was defined as total intake. The percentage of sucrose intake was calculated according to the following equation: % sucrose preference = (sucrose intake / total intake) × 100. The tests began 1 week prior to neurotoxin exposure to provide baseline values and were completed at time-point 14 days.

### *TH Immunohistochemistry*

Rats were deeply anesthetized with ketamine immediately after the behavior tests, and were intracardially perfused with saline first, then with 4% of the fixative solution formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and were immersed for 1 week in that fixative solution at 4°C. Subsequently, the brains were placed in 30% sucrose solution for 3 days and were freeze at -80°C before sectioning. Six 40 µm sections per animal were taken from the olfactory bulb (+7.56 mm and +7.08 mm an interval of 480 µm) and six sections per animal were taken from the SNpc (with an interval of 360 µm): bregma -4.92 mm and -5.28 mm. These sections were chosen because of their location in the mid-rostrocaudal part of the substantia nigra, which contains many dopaminergic neurons. Tissue sections were incubated with primary mouse anti-TH antibody, diluted in phosphate-buffered saline containing 0.3% Triton X-100 (1:1.000 in SNpc sections and 1:8.000 in OB sections; cat # AB152 Chemicon, CA, USA) overnight at 4°C. Biotin-conjugated secondary antibody incubation (1:200 in both structures cat # S-1000 Vector Laboratories, USA), was performed for 2h at room temperature. After several washes in phosphate-buffered saline, antibody complex was localized using the ABC system (Vectastain ABC Elite kit cat # PK6101, Vector Laboratories, USA)

followed by 3,3'-diaminobenzidine reaction with nickel enhancement. The sections were then mounted onto gelatin-coated slides and coverslipped after dehydration in ascending concentrations of ethanol-xylene solutions. OB manual cell counts and SNpc neuronal density determination were conducted making use of the softwares Image-Pro Express 6 and Image-J, respectively. The mean number of TH-ir neurons in each hemisphere was considered to be representative of the SNpc neuronal cells in each animal. For each group, a mean value was calculated and compared with those of the other groups. The images were obtained using a motorized Axio Imager Z2 microscope (Carl Zeiss, Jena, DE), equipped with an automated scanning VSlide (Metasystems, Altlussheim, DE).

### *Statistical analysis*

Differences between groups in the ODT were analyzed by two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Olfactory discrimination index, modified forced swimming test, open-field test and TH-immunohistochemistry were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. Sucrose preference test was analyzed by two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. Pearson's correlation coefficients ( $r$ ) were calculated to establish relationships between histological and behavioral parameters. Values were expressed as mean  $\pm$  standard error of mean (SEM). The level of significance was set at  $P \leq 0.05$ .

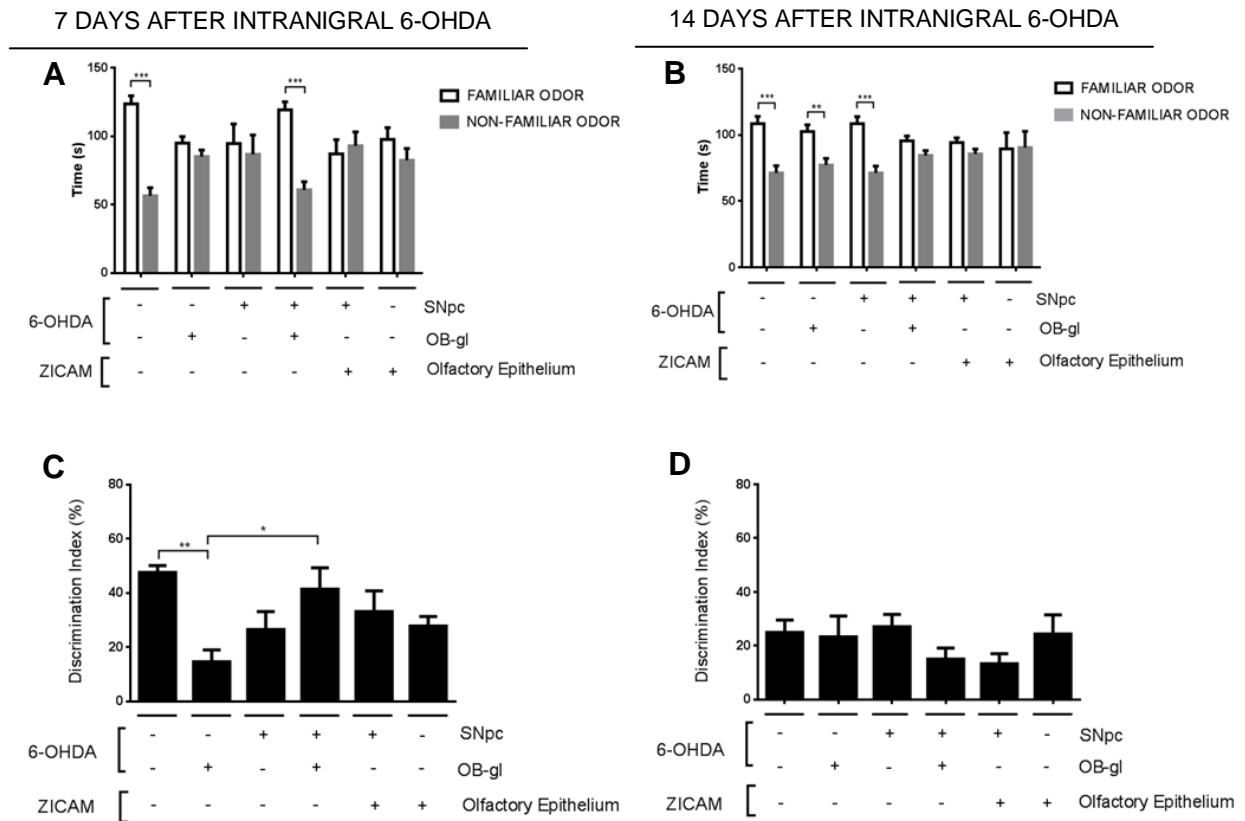
## **Results**

### *Olfactory Discrimination Task (ODT)*

We have found, at the time-point 7 days, that the group 6-OHDA (-)SNpc/(-)OB-gf showed a significant exploration time ( $P \leq 0.001$ ) of the familiar odor compared to non-familiar. Similarly, the double-lesioned group 6-OHDA (+)SNpc/(+)OB-gf, demonstrated an increased exploration of the familiar odor ( $P \leq 0.001$ ) (Fig. 2A). In opposite, the 6-OHDA (-)SNpc/(+)OB-gf ( $P > 0.99$ ), as well as the 6-OHDA (+)SNpc/(-)OB-gf groups ( $P > 0.99$ ) and the positive controls for anosmia 6-

OHDA (+)SNpc/(-)OB-gl/(+)Zicam ( $P > 0.99$ ) and 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P > 0.99$ ) exhibited similar exploration times for both odors, according to the lesion [ $F(5, 86) = 0.001$ ;  $P > 0.99$ ], odor [ $F(1, 86) = 24.97$ ;  $P < 0.0001$ ] and interaction [ $F(5, 86) = 5.868$ ;  $P = 0.0001$ ] factors (Fig. 2A). The analysis of the time-point 14 days demonstrated that the groups 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.001$ ), 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.01$ ), and 6-OHDA (+)SNpc/(-)OB-gl ( $P \leq 0.0001$ ) spent significantly less time exploring the non-familiar odor compared to the familiar, as indicated by the odor [ $F(1, 86) = 24.97$ ;  $P \leq 0.0001$ ], lesion [ $F(5, 96) = 3.724$ ;  $P > 0.99$ ] and interaction [ $F(5, 86) = 5.868$ ;  $P = 0.0001$ ] factors (Fig. 2B). However, we did not find differences between exploration time between odors for the groups 6-OHDA (+)SNpc/(+)OB-gl ( $P = 0.73$ ) and also for the positive controls for anosmia 6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam ( $P > 0.99$ ) and 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P > 0.99$ ) (Fig. 2B).

Figures 2C and 2D show the DIs obtained from the ODT at time-points 7 and 14 days, respectively. The group 6-OHDA (-)SNpc/(+)OB-gl exhibited a significant impairment in the DI compared to the 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) and the 6-OHDA (+)SNpc/(+)OB-gl ( $P \leq 0.01$ ) as indicated by the multiple comparisons [ $F(5, 46) = 4.155$ ;  $P = 0.0034$ ] (Fig. 2C). Notwithstanding, at time-point 14 days, we did not observe changes in the DI among the groups [ $F(5, 53) = 1.393$ ;  $P = 0.2419$ ] (Fig. 2D).



**Figure 2. (A) and (B)** Time (s) spent in familiar and non-familiar compartments in the olfactory discrimination task (ODT) 7 and 14 days after 6-OHDA intranigral microinfusion, respectively. The bars represent the mean  $\pm$  standard error of the mean.  $n = 12$  per group,  $**P \leq 0,01$ ,  $***P \leq 0,001$  comparing the mean time spent in familiar and non-familiar compartments. Two-way ANOVA followed by the Bonferroni post hoc test. **(C) and (D)** Olfactory discrimination index (DI) – from A and B, respectively.  $DI = (NF - F / NF + F) * 100$ , NF is the time spent in the compartment with non-familiar odor and F is the time spent in the compartment with familiar odor. The bars represent the mean  $\pm$  standard error of the mean,  $n = 12$  per group,  $*P \leq 0,05$ ,  $**P \leq 0,01$ . One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (-) absence of 6-OHDA lesion or Zicam (sham manipulation).

### Modified forced swimming test

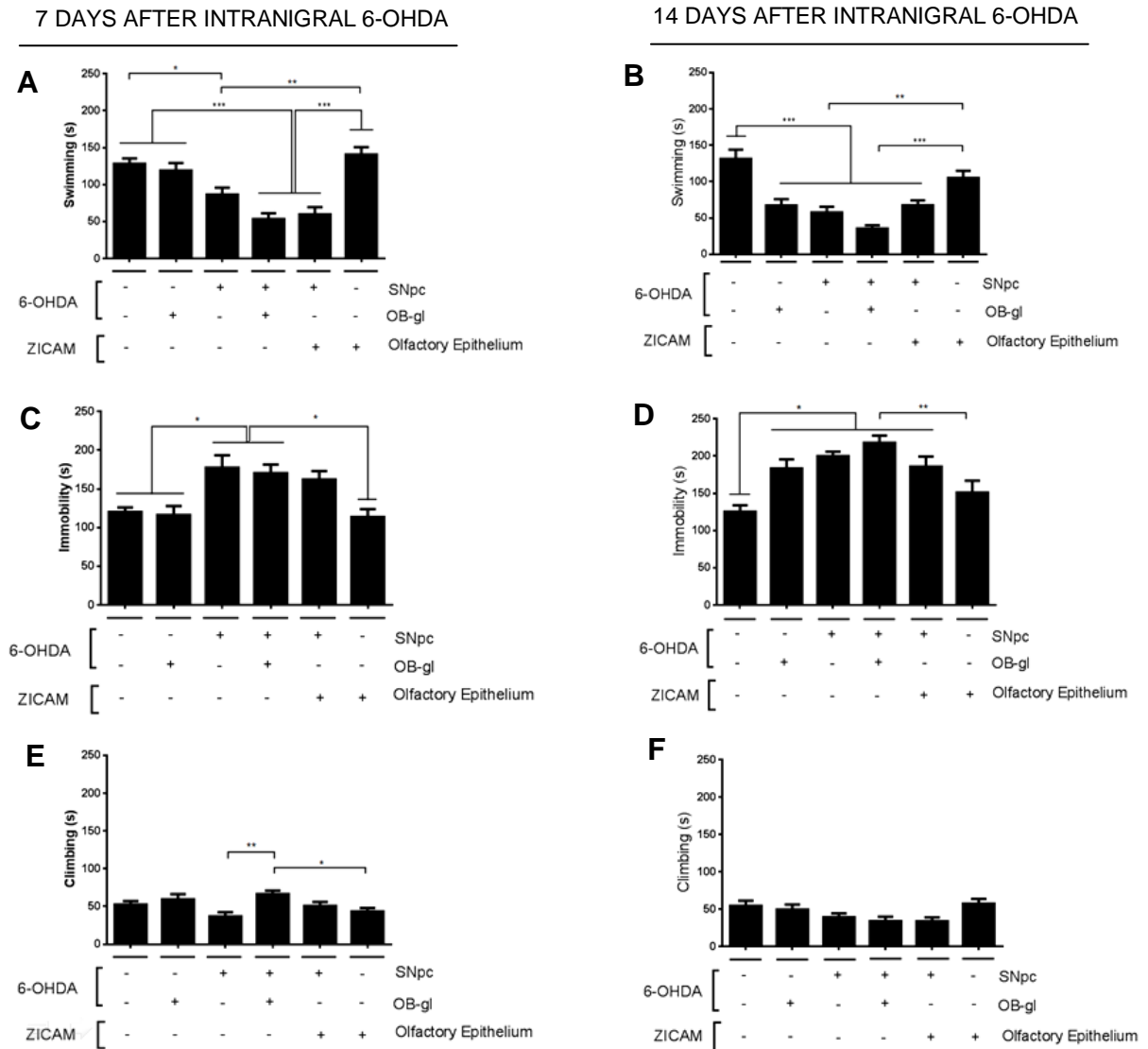
The results from time-point 7 days evidenced a significant reduction of the swimming time in the group 6-OHDA (+)SNpc/(-)OB-gl when compared to 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) group and the positive control for anosmia 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.01$ ) group (Fig. 3A). Also, the double 6-OHDA lesion group, i.e., 6-OHDA (+)SNpc/(+)OB-gl presented a significant reduction of swimming time compared to the 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.001$ ) and 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.001$ ) groups, as well as for the positive control for anosmia 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.001$ ), [ $F(5, 49) = 16.62$ ;  $P < 0.0001$ ] (Fig. 3A). The examination of the same parameter at time-point 14 days showed that all 6-OHDA lesioned groups, that is, 6-OHDA (-)SNpc/(+)OB-gl, 6-OHDA (+)SNpc/(-)OB-gl, 6-OHDA (+)SNpc/(+)OB-gl and 6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam presented a



similar reduction ( $P < 0.001$ , for all groups) when compared to 6-OHDA (-)SNpc/(-)OB-gl (Fig. 3B). Furthermore, the groups 6-OHDA (+)SNpc/(-)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl exhibited reductions in this parameter; ( $P \leq 0.01$ ) and ( $P \leq 0.001$ ), respectively, in comparison to the control for anosmia 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam group, [ $F(5.49) = 17.45$ ;  $P \leq 0.0001$ ] (Fig. 3B).

As depicted in Fig. 3C, the immobility time of the groups 6-OHDA (+)SNpc/(-)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl (at time-point 7 days) appears to be equally increased in comparison to the 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) and 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.01$ ) groups. In addition, such increment in immobility was also observed in comparison to the control for anosmia 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam group ( $P \leq 0.05$ ), [ $F(5.48) = 6.665$ ;  $P \leq 0.0001$ ]. Figure 3D represents the immobility time obtained at time-point 14 days. It is noticeable that all the 6-OHDA-lesioned groups, that is, 6-OHDA (-)SNpc/(+)OB-gl, 6-OHDA (+)SNpc/(-)OB-gl, 6-OHDA (+)SNpc/(+)OB-gl and 6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam present increased times of immobility when compared to the controls 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) and 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.001$ ), [ $F(5.55) = 7.989$ ;  $P \leq 0.0001$ ].

Complementarily, the climbing time of the groups, at time-point 7 days, showed to be increased in the 6-OHDA (+)SNpc/(+)OB-gl when compared to the 6-OHDA (+)SNpc/(-)OB-gl ( $P \leq 0.01$ ) and the positive control for anosmia 6-OHDA (-)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.05$ ), [ $F(5.41) = 3.948$ ;  $P = 0.0052$ ] (Fig. 3E). Moreover, the analysis of the time-point 14 days revealed significant differences in ANOVA among groups [ $F(5.47) = 2.560$ ;  $P = 0.0396$ ], but did not showed it in multiple comparisons (Fig. 3F).

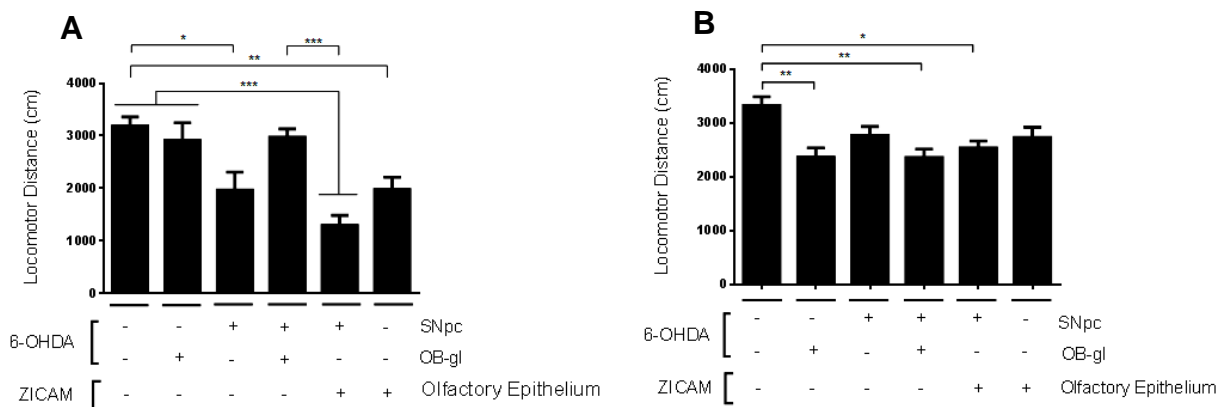


**Figure 3.** Depressive-like behaviors during the modified forced swimming test. **(A)** swimming time 7 days after intranigral 6-OHDA, **(B)** swimming time 14 days after intranigral 6-OHDA, **(C)** immobility time 7 days after intranigral 6-OHDA, **(D)** immobility time 14 days after intranigral 6-OHDA, **(E)** climbing time 7 days after intranigral 6-OHDA, **(F)** climbing time 14 days after intranigral 6-OHDA. The bars represent the mean  $\pm$  standard error of the mean.  $n = 12$  per group, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . **(C)** The \* $P$  for (-)SNpc/(-)OB-gl represent \* $P \leq 0.05$  vs. (+)SNpc/(-)OB-gl and \* $P \leq 0.05$  vs. (+)SNpc/(+)OB-gl. The \* $P$  for (-)SNpc/(+)OB-gl and (-) SNpc/(+)Olfactory Epithelium represents \* $P \leq 0.05$  vs. (+)SNpc/(+)OB-gl and \*\* $P \leq 0.01$  vs. (+)SNpc/(-)OB-gl for both groups. **(D)** The \* $P$  for (-)SNpc/(-)OB-gl represent \* $P \leq 0.05$  vs. (-)SNpc/(+)OB-gl \*\*\* $P \leq 0.001$  vs. (+)SNpc/(-) OB-gl \*\*\* $P \leq 0.001$  (+)SNpc/(+)OB-gl, \*\* $P \leq 0.01$  (+) SNpc/(+)Olfactory Epithelium. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (-) absence of 6-OHDA lesion or Zicam (sham manipulation).

### Open-field (OF) test

In the open-field test at time-point 7 days (Fig. 4A), significant reductions in the spontaneous locomotor behavior were observed in the 6-OHDA (+)SNpc/(-)OB-gl group ( $P \leq 0.05$ ) and also in the two positive controls for anosmia 6-OHDA (-) SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.001$ ) and 6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.0001$ ) compared to the 6-OHDA (-)SNpc/(-)OB-gl. In addition, the locomotion of the group

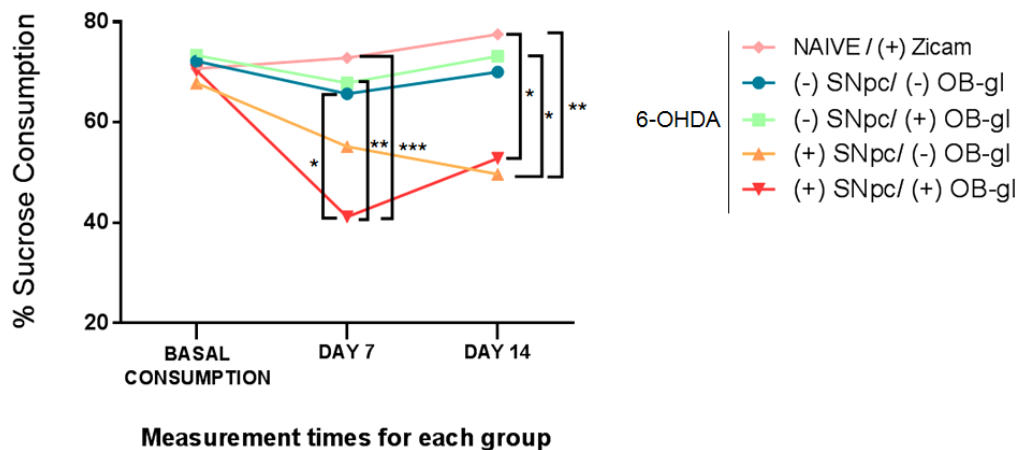
6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam was also reduced compared to 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.001$ ) and 6-OHDA (+)SNpc/(+)OB-gl ( $P \leq 0.001$ ), [ $F(5, 44) = 9.618$ ,  $P < 0.0001$ ]. Similarly, at time-point 14 days (Fig. 4B) reductions in locomotion were detected for the groups 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.01$ ), (+)SNpc/(+)OB-gl ( $P \leq 0.01$ ) and positive control for anosmia 6-OHDA (+)SNpc/(-)OB-gl/(+)Zicam ( $P \leq 0.05$ ) compared to the 6-OHDA (-)SNpc/(-)OB-gl group, [ $F(5, 56) = 4.535$ ,  $P = 0.0015$ ].



**Figure 4.** Locomotor distance in the open-field test (OF) 7 days after intranigral 6-OHDA (A) and 14 days after intranigral 6-OHDA (B). The bars represent the mean  $\pm$  standard error of the mean.  $n = 12$  per group, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ . One-way ANOVA followed by the Newman-Keuls post hoc test. : 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (-) absence of 6-OHDA lesion or Zicam (sham manipulation).

### Sucrose Preference Test

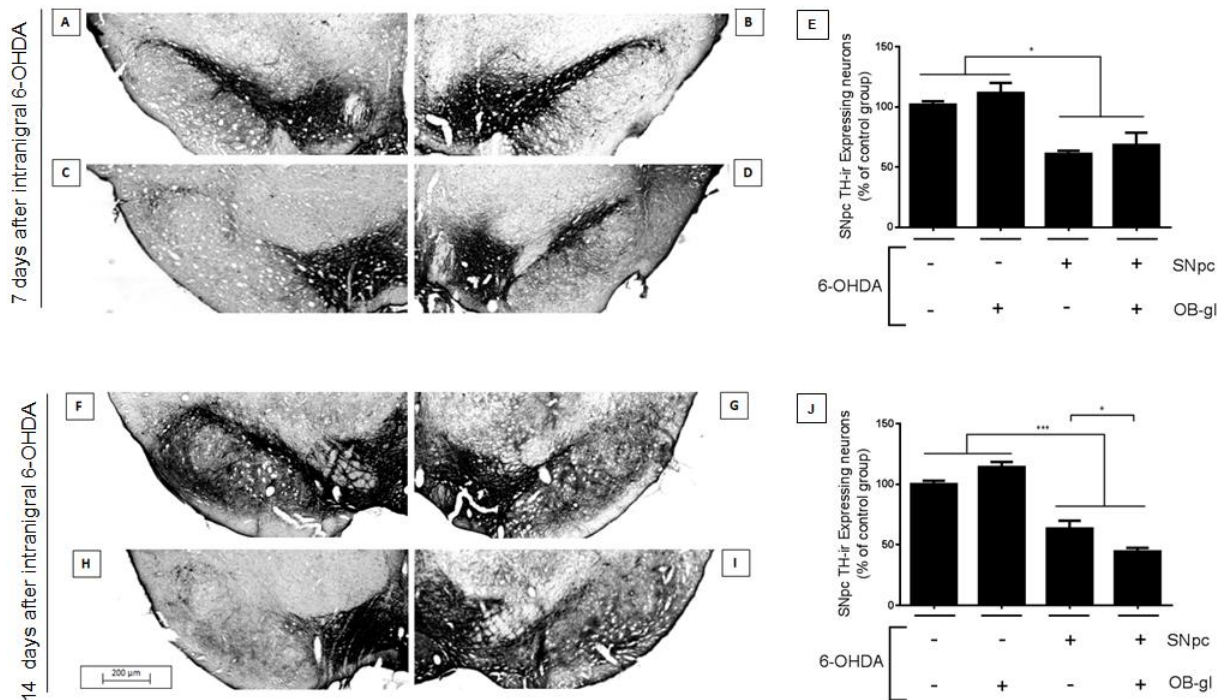
The analysis of sucrose preference (Fig. 5) revealed that only 6-OHDA double-lesioned group, i.e., 6-OHDA (+)SNpc/(+)OB-gl exhibited a significant reduction of sucrose consumption compared to three groups: Naive/(+)Zicam ( $P \leq 0.001$ ), 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) and 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.01$ ) for the time-point 7 days. At 14 days, the anhedonic effect was still detected in the 6-OHDA (+)SNpc/(+)OB-gl group compared to Naive/(+)Zicam ( $P \leq 0.05$ ). At the same time-point, the group 6-OHDA (+)SNpc/(-)OB-gl also presented a significant reduction of sucrose consumption compared to Naive/(+)Zicam ( $P \leq 0.01$ ) and 6-OHDA (-)SNpc/(+)OB-gl; ( $P \leq 0.05$ ), according to the lesion [ $F(4, 25) = 6.639$ ;  $P = 0.0009$ ], time-point [ $F(2, 50) = 6.537$ ;  $P = 0.0030$ ] and interaction [ $F(8, 50) = 2.413$ ;  $P = 0.0275$ ] factors.



**Figure 5.** Percentage of the sucrose consumption measured in the Sucrose Preference Test (SPT). The symbols represent the mean.  $n = 8$  per group.  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$ . Two-way ANOVA with repeated measures followed by the Bonferroni post hoc test. A Naïve (non-operated) group was included and only received intranasal Zicam. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion or Zicam administration; (-) absence of 6-OHDA lesion (sham manipulation).

### *TH Immunohistochemistry within the SNpc*

The analysis of the dopaminergic neurons in the SNpc revealed that 6-OHDA caused a pronounced neuronal loss at both time-points (see panels at Fig.6). Figure 6E shows the quantification of TH-ir neurons density at time-point 7 days revealing that 6-OHDA (+)SNpc/(-)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl exhibited significant reductions ( $P \leq 0.001$ ) ( $P \leq 0.05$ ), respectively, compared to the control 6-OHDA (-)SNpc/(-)OB-gl group. The same groups also exhibited significant decrease in TH-ir neurons compared to the 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.0001$ ) and ( $P \leq 0.001$ ) respectively, [ $F(3, 49) = 17.26$ ;  $P < 0.0001$ ]. Likewise, 14 days time-point observation (Fig. 6J), demonstrated that the same groups with 6-OHDA SNpc lesion; 6-OHDA (+)SNpc/(-)OB-gl and 6-OHDA (+)SNpc/(+)OB-gl, exhibited significant reductions in this parameter compared to the control group 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.0001$  for both groups) and compared to the 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.0001$  for both groups as well). Remarkably, 6-OHDA (+)SNpc/(+)OB-gl demonstrated a further decrement in nigral TH-ir neurons compared to the 6-OHDA (+)SNpc/(-)OB-gl ( $P \leq 0.05$ ), as indicated [ $F(3, 62) = 50.25$ ;  $P \leq 0.0001$ ].

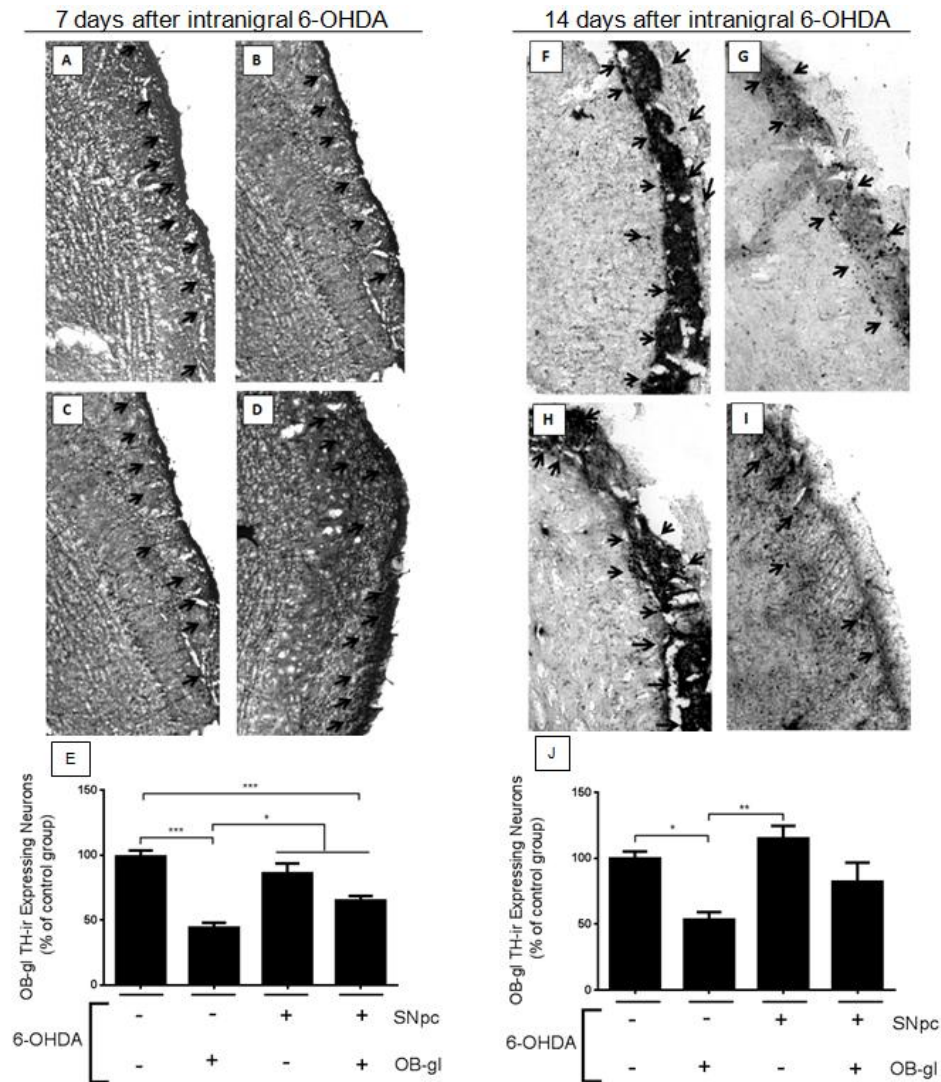


**Figure 6.** Immunohistochemistry of TH-ir neurons in the SNpc. Panels A, B, C and D show representative photomicrographies obtained 7 days after intranigral 6-OHDA microinfusion, Panel E is the quantification of SNpc TH-ir neurons 7 days after 6-OHDA. Panels F, G, H and I show representative photomicrografies obtained 14 days after intranigral 6-OHDA. Panel J is the quantification of SNpc TH-ir neurons 14 days after 6-OHDA. Painels A and F: (-)SNpc/(-)OB-gl groups; painels B and G: (-)SNpc/(+)OB-gl groups; painels C and H: (+)SNpc/ (-)OB-gl groups; painels D and I: (+)SNpc/(+)OB-gl groups. The control groups for olfactory impairment (ZICAM) were not included. The bars represent the mean  $\pm$  standard error of the mean.  $n = 24$  sections (4 animals, 6 slices each) per group,  $*P \leq 0.05$ ,  $***P \leq 0.001$ . (E)  $*P$  represent [ $*P \leq 0.05$  vs. (+)SNpc/(+)OB-gl;  $***P \leq 0.001$  vs (+)SNpc/(-)OB-gl]. One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion; (-) absence of 6-OHDA lesion (sham manipulation).

### *TH Immunohistochemistry within the OB-gl*

The dopaminergic neuronal population in the glomerular layer of the OB is represented in Figure 7. As can be seen in Fig. 7E (time-point 7 days), it is indicated a significant decrease in TH-ir neurons in the group 6-OHDA (-) SNpc/(+)OB-gl compared to the groups 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.0001$ ) and 6-OHDA (+)SNpc/(-)OB-gl ( $P \leq 0.0001$ ). Furthermore, 6-OHDA (+)SNpc/(+)OB-gl group also exhibited a significant neuronal reduction compared to 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.001$ ). However, this 6-OHDA (+)SNpc/(+)OB-gl group presented an increased density of TH-ir neurons compared to the 6-OHDA (-)SNpc/(+)OB-gl ( $P \leq 0.05$ ), [ $F(3, 22) = 28.23$ ;  $P \leq 0.0001$ ]. In relation to the time-point 14 days (Fig. 7J), the 6-OHDA (-)SNpc/(+)OB-gl group showed a significant reduction of this parameter compared to the 6-OHDA (-)SNpc/(-)OB-gl ( $P \leq 0.05$ ) and 6-OHDA (+)SNpc/(-)OB-gl ( $P \leq 0.01$ ) groups, [ $F(3, 16) = 6.428$ ;  $P = 0.0046$ ].





**Figure 7.** Immunohistochemistry of TH-ir neurons in the OB. The black arrows indicate TH-ir neurons at the glomerular layer. Panels A, B, C and D show representative photomicrographies obtained 7 days after intranigral 6-OHDA microinfusion. Panel E is the quantification of OB-gl TH-ir neurons 7 days after 6-OHDA. Panels F, G, H and I show representative photomicrographies obtained 14 days after intranigral 6-OHDA. Panel J is the quantification of OB-gl TH-ir neurons 14 days after 6-OHDA. Panels A and F: (-)SNpc/(-)OB-gl groups; panels B and G: (-)SNpc/(+)OB-gl groups; panels C and H: (+)SNpc/(-)OB-gl groups; panels D and I: (+)SNpc/(+)OB-gl groups. The control groups for olfactory impairment (Zicam) were not included. The bars represent the mean  $\pm$  standard error of the mean.  $n = 24$  sections (4 animals, 6 slices each) per group, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  One-way ANOVA followed by the Newman-Keuls post hoc test. Legends: 6-OHDA, 6-hydroxydopamine; OB-gl, olfactory bulb glomerular layer; SNpc, substantia nigra pars compacta; (+) presence of 6-OHDA lesion; (-) absence of 6-OHDA lesion (sham manipulation).

### *Statistical correlations between behavioral and histological parameters*

Pearson's correlation coefficients (Table 1) revealed a negative correlation between the percentage of SNpc TH-ir neurons and immobility parameter of modified forced swimming test at time-points 7 ( $r = -0.51$ ,  $P = 0.002$ ) and 14 days ( $r = -0.60$ ,  $P < 0.0001$ ). Proportionally, a positive correlation between the percentage of SNpc TH-ir neurons and swimming parameter from the modified forced swimming test was

found also at 7 ( $r = 0.53$ ,  $P = 0.001$ ) and 14 days ( $r = 0.52$ ,  $P = 0.0006$ ). The climbing parameter also showed a positive correlation with the percentage of SNpc TH-ir neurons, but only at 14 days ( $r = 0.44$ ,  $P = 0.005$ ). In addition, positive correlations between the percentage of OB-gl TH-ir neurons and DI were identified at 7 ( $r = 0.59$ ,  $P = 0.002$ ) and 14 days ( $r = 0.45$ ,  $P = 0.03$ ). Complementarily, the percentage of sucrose consumption exhibited a negative correlation with immobility time in both time-points: 7 ( $r = -0.51$ ,  $P = 0.01$ ) and 14 days ( $r = -0.44$ ,  $P = 0.02$ ), positive correlation with swimming time at 7 ( $r = 0.45$ ,  $P = 0.02$ ) and 14 days ( $r = 0.52$ ,  $P = 0.008$ ) and a positive correlation with the percentage of SNpc TH-ir neurons ( $r = 0.09$ ;  $P = 0.67$  time-point 7 days) ( $r = -0.055$ ;  $P = 0.82$  time-point 14 days). Moreover, the percentage of OB-gl TH-ir neurons exhibited a negative correlation with immobility time ( $r = -0.44$ ,  $P = 0.04$ ) and a positive correlation with swimming time ( $r = 0.48$ ,  $P = 0.02$ ) but only at 14 days. Ultimately, correlations between percentage of SNpc TH-ir neurons and percentage of OB-gl TH-ir neurons were not found.

**Table 1. Pearson's correlations between different behavioral and histological parameters.**

<b>CORRELATIONS</b>	<b>7 days after intranigral 6-OHDA groups</b>	<b>14 days after intranigral 6-OHDA groups</b>
% SNpc TH-ir Neurons x % OB-gl TH-ir Neurons	$r = -0,35$ ; $P = 0,08$	$r = 0,054$ ; $P = 0,82$
% SNpc TH-ir Neurons x DI	$r = -0,003$ ; $P = 0,98$	$r = 0,2017$ ; $P = 0,19$
% SNpc TH-ir Neurons x Immobility	$r = -0,51$ ; $P = 0,002^*$	$r = -0,60$ ; $P < 0,0001^*$
% SNpc TH-ir Neurons x Swimming	$r = 0,53$ ; $P = 0,001^*$	$r = 0,52$ ; $P = 0,0006^*$
% SNpc TH-ir Neurons x Climbing	$r = 0,19$ ; $P = 0,28$	$r = 0,44$ ; $P = 0,005^*$
% OB-gl TH-ir Neurons x DI	$r = 0,59$ ; $P = 0,002^*$	$r = 0,45$ ; $P = 0,03^*$
% OB-gl TH-ir Neurons x Immobility	$r = -0,059$ ; $P = 0,79$	$r = -0,44$ ; $P = 0,04^*$
% OB-gl TH-ir Neurons x Swimming	$r = 0,11$ ; $P = 0,62$	$r = 0,48$ ; $P = 0,02^*$
% OB-gl TH-ir Neurons x Climbing	$r = -0,30$ ; $P = 0,15$	$r = -0,35$ ; $P = 0,12$
% Sucrose Consumption x DI	$r = -0,10$ ; $P = 0,63$	$r = 0,26$ ; $P = 0,21$
% Sucrose Consumption x Immobility	$r = -0,51$ ; $P = 0,01^*$	$r = -0,44$ ; $P = 0,02^*$
% Sucrose Consumption x Swimming	$r = 0,45$ ; $P = 0,02^*$	$r = 0,52$ ; $P = 0,008^*$
% Sucrose Consumption x % SNpc TH-ir Neurons	$r = 0,43$ ; $P = 0,03^*$	$r = 0,45$ ; $P = 0,02^*$
% Sucrose Consumption x % OB-gl TH-ir Neurons	$r = 0,09$ ; $P = 0,67$	$r = -0,055$ ; $P = 0,82$

\* Significant correlations are indicated.

## Discussion

In the present study we observed at the first time-point, 7 days after intranigral 6-OHDA microinfusion, that SNpc and OB-gl dopaminergic lesions were able to disrupt olfaction like Zicam in a very similar fashion. It has been reported that an increased number of TH-ir neurons within the glomerular layer found in PD in post-mortem brains (Huisman et al., 2004; Mundiñano et al., 2011) and rotenone animal model of PD (Rodrigues et al., 2014) may be associated to the olfactory impairment. Curiously, at the earlier time-point, the group 6-OHDA (+)SNpc/(-)OB-gl did not present increase in TH-ir neurons in the OB-gl compared to the control group. Therefore, this olfactory impairment without periglomerular cells density increment corroborate with previous findings from Höglinger and colleagues (2015), that also did not find increase of dopaminergic neurons in OB-gl after 6-OHDA intranigral in rats. Moreover, results from Huisman and colleagues (2008) showed an even more puzzling result that disputed the so called TH-ir neuronal increase in OB-gl in humans as found in their previous study (Huisman et al., 2004). In fact, our results show that the olfactory impairment presented in the OB-gl-lesioned group may be associated to periglomerular TH-ir neuronal loss compared to control. In this sense, our result stands that TH-ir periglomerular neurons are responsible for the olfactory modulation thus, a decrease of these cells density negatively impacts olfactory performance, as previously demonstrated (Doty, 2012; Mundiñano et al., 2011). In relation to the double-site 6-OHDA infusion procedure, the OB-gl lesion seems to reverse the olfactory impairment caused by SNpc lesion, possibly as a consequence of the decline in the increment of the dopaminergic neurons within the OB-gl.

In opposite, at time-point 14 days, both groups selectively lesioned in the SNpc and in the OB-gl did not present detectable olfactory impairments, possibly as a result of compensatory mechanisms of the main olfactory pathway. Considering the OB-gl-lesioned group, we found that TH-ir periglomerular neurons were still decreased compared to the control and SNpc- lesioned group, however, not differing from the double-lesioned group. Remarkably, neurotoxic-induced lesions within the OB of mice is recovered by an increase of the newborn neurons coming from subventricular zone (SVZ), where they are stem cells (Hoglinger et al., 2004; Tieu, 2011). Such cells are recruited and then migrate through rostral pathway only differentiating in dopaminergic and GABAergic interneurons at glomerular and



granular layers, approximately 7 days post 6-OHDA injury (Lazarini *et al.*, 2014). Therefore, these newborn neurons are able to repopulate (at least partially) the OB-gl, then recovering the olfactory sensory inputs. In fact, it is described that 8 days after the 6-OHDA OB-gl lesion, animals could recover smell sense due to compensatory mechanisms, p.e. through activation of vomeronasal pathway and accessory olfactory system (Salazar *et al.*, 2015). The vomeronasal pathway is an alternative olfactory pathway described as a primitive system found in several mammals (Savic *et al.*, 2009; Trotier, 2011), but in humans the system is present only in fetus (Salazar *et al.*, 2015). In rodents, vomeronasal pathway was first reported as able to identify pheromones and common odors were perceived through the olfactory pathway (main olfactory system), but nowadays this concept has been questioned (Keverne, 2005; Shepherd, 2006 *apud* Salazar *et al.*, 2015).

Regarding the extension of the nigral lesion, the percentage of TH-ir neurons, at time-point 7 days, was decreased only in association with intranigral 6-OHDA, as previously reported (Kirik *et al.*, 1998; Santiago *et al.*, 2014; Penttinen *et al.*, 2016; Fricke *et al.*, 2016; Matheus *et al.*, 2016), without being influenced by OB-gl injury. However, the later time-point showed the occurrence of a possible synergic effect, due to a conceivable retrograde OB-gl lesion, impacting SNpc. In fact, this hypothesis is supported by the description of a direct axonal dopaminergic projection from SNpc to the extern plexiform layer and granular layer of the OB, which promotes the perception of odorants and can mediate toxin-induced retrograde degeneration of dopaminergic SNpc neurons (Höglinger *et al.*, 2015). In our study, we suggest that the maturity of the OB-gl lesion could be responsible for expand the injury, reaching nigrobulbar projection and increasing the SNpc neuron loss. The absence of differences between TH-ir neurons in OB-gl of double-lesioned group and control group also (also in opposition of time-point 7 days) may be interpreted as a compensatory increase in the number of the periglomerular neurons from OB-gl, possibly explaining the olfactory impairment, in agreement with our previous findings (Rodrigues *et al.*, 2014). Of note, in this study we demonstrated that the olfactory impairment at 7 and 14 days were particularly alike than the olfactory deficit inflicted by intranasal Zicam, which was used as a positive control of anosmia. This is an agent that has been described to promote a significant cytotoxicity to human, mouse and rats nasal tissue given the potential development of long-lasting smell dysfunction (Lim *et al.*, 2009; Chioca *et al.*, 2013; Rodrigues *et al.*, 2014). Further,

both positive controls for anosmia exhibited hipolocomotion at 7 days. This result could be related to a residual anesthetic effect due to intranasal Zicam administration process, however, ketamine is supposed to produce hyperlocomotion in similar conditions (Hetzler and Wautlet, 1985; Wilson et al., 2007; Radford et al., 2017). Notwithstanding, this hipolocomotion was reversed at 14 days, suggesting an absence of locomotor bias.

As formerly described, intranigral 6-OHDA causes increase in the immobility time and consequent decreases swimming time and also reduces sucrose consumption at 7 (Tadaiesky et al., 2008; Matheus et al., 2016; Chiu et al., 2015), 14 (Santiago et al., 2010; Santiago et al., 2014; Furlanetti et al., 2015) and other time-points already tested (Santiago et al., 2015; Zhang et al., 2015). Remarkably, double lesion procedure produced depressive-like behaviors, most likely as decreased swimming, increased immobility and anhedonic-like behavior at both time-points tested. This outcome is also interesting when compared to OB-gl lesion itself that only produced behavior despair, without anhedonia, at the later time-point, strengthen the notion of a maturation process of the retrograde lesion. Depression causes alterations in olfactory circuits, reducing olfactory threshold, identification and discrimination abilities in humans (Atanasova et al., 2010; Negoias et al., 2010). Analogously, patients with congenital anosmia are more expected to exhibit signs of depression (Croy et al., 2012).

Our study originally demonstrated that a dopaminergic lesion of the OB-gl was able to produce depressive-like behaviors perhaps as a result of dysfunctions and/or compensatory mechanisms of the cortical–hippocampal–amygdala circuits that involve changes in synaptic strength and/or loss of spine density in these limbic areas (Price and Drevets, 2012 apud Czéh et al., 2016). Besides, we found significant correlations between OB-gl TH-ir neuronal count and immobility time ( $r = -0,44$ ;  $P = 0,04$ ) and swimming time ( $r = 0,48$ ;  $P = 0,02$ ) at the later time-point, suggesting a direct association between these TH-ir periglomerular neurons and depressive-like behaviors. A correlation between OB-gl TH-ir neuronal count and DI of olfactory test is observed (at 7 days  $r = 0,59$ ;  $P = 0,002$  and at 14 days  $r = 0,45$ ;  $P = 0,03$ ). At both time-points, the reduction of dopaminergic neurons of OB-gl indicates a decrement of DI, suggesting the role of these periglomerular dopaminergic neurons within the OB-gl as keyplayers to allow the proper functioning of the olfactory system. In accordance, the inhibition of the olfactory epithelium did not cause depressive-like

behaviors such as helplessness and despair or anhedonia. Despite the absence of correlations between DI and depressive-like parameters, our results reinforce the importance of the dopaminergic neurons of OB-gl for both PD non-motor symptoms, since the precisely reduction of the periglomerular interneurons was able to induce olfactory impairment (at acute time-point), helplessness and despair behaviors (at later time-point). At the same time, the acute OB-gl lesion was able to reverse the olfactory impairment caused by SNpc injury to mimic PD and not able to cause depressive-like behaviors. To clarify the relation between PD, depression and olfactory impairment, more studies will be necessary to identify the role of these dopaminergic neurons of the OB-gl with dopamine neurotransmitter quantification, and the neuronal amount of limbic structures to expand the knowledge of the relation to these non-motor symptoms with or without parkinsonism context.

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## 5. CONCLUSÕES

- Em relação à olfação, observamos que as lesões com 6-OHDA na SNpc e no BO, isoladamente, promoveram notável prejuízo olfatório na análise de 7 dias. Já a lesão no BO foi capaz de reverter o prejuízo olfatório causado pela lesão intranigral, o que indica a influência dos interneurônios periglomerulares na transmissão olfatória.
- A lesão com 6-OHDA intranigral mimetizou o comportamento tipo-depressivo no TNFM, nos dois tempos testados, e no TPS (na avaliação de 14 dias). A lesão no BO reproduziu o efeito tipo-depressivo na linha temporal de 14 dias no teste de natação forçada modificado, não sendo observado o comportamento anedônico. As lesões em associação demonstraram efeito sinérgico em relação à anedonia.
- O grupo com dupla lesão apresentou maior perda neuronal na SNpc comparado ao grupo somente lesionado na mesma, demonstrando uma possível lesão retrógrada que pode ser correlacionada à piora dos sintomas depressivos. O grupo duplamente lesionado apresentou uma redução menor nos neurônios do BO comparado ao grupo que foi lesionado só no bulbo, indicando algum mecanismo de compensação, corroborado pelos testes comportamentais.

Apesar da ausência de correlação entre DI e parâmetros depressivos, nossos resultados reforçam a importância dos neurônios dopaminérgicos do OB-gl para ambos os sintomas não-motores de DP, uma vez que a redução precisa dos interneurônios periglomerulares foi capaz de induzir comprometimento olfatório (em 7 dias), desamparo e comportamentos de desespero (em 14 dias).

- Estes resultados contribuem para aumentar o entendimento do papel do sistema dopaminérgico em relação aos sintomas não-motores da DP e como o estudo de dois sintomas tão distintos como prejuízo olfatório e depressão podem estar intrinsecamente relacionados.

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